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PCT

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(54) Title: THERAPEUTIC USES OF BACTERICIDAL/PERMEABILITY INCREASING PROTEIN PRODUCTS		
<p>(57) Abstract</p> <p>The present invention provides therapeutic methods for treatment of conditions including the neutralization of the anti-coagulant activity of heparin, inhibition of angiogenesis, tumor and endothelial cell proliferation, and treatment of chronic inflammatory diseases by administration of bactericidal/permeability-increasing (BPI) protein products.</p>		

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THERAPEUTIC USES OF BACTERICIDAL/PERMEABILITY INCREASING PROTEIN PRODUCTS

This is a continuation-in part of U.S. Patent Application Serial
5 No. 08/030,644 filed March 12, 1993.

BACKGROUND OF THE INVENTION

The present invention relates to therapeutic uses of
bactericidal/permeability increasing (BPI) protein products for the treatment of
conditions related to gram-negative bacterial infection and the conditions not
10 directly associated with gram-negative bacterial infection, including
neutralization of the anti-coagulant properties of heparin, inhibition of
angiogenesis, tumor and endothelial cell proliferation and treatment of chronic
inflammatory disease states such as arthritis.

Heparin Binding

15 Heparin is a heterogenous group of straight-chain anionic
mucopolysaccharides, called glycosaminoglycans having anticoagulant
properties. Although others may be present, the main sugars occurring in
heparin are: (1) α -L-iduronic acid 2-sulfate, (2) 2-deoxy-2-sulfamino- α -D-
glucose 6-sulfate, (3) β -D-glucuronic acid, (4) 2-acetamido-2-deoxy- α -D-
20 glucose, and (5) α -L-iduronic acid. These sugars are present in decreasing
amounts, usually in the order (2) > (1) > (4) > (3) > (5), and are joined by
glycosidic linkages, forming polymers of varying sizes. Heparin is strongly
acidic because of its content of covalently linked sulfate and carboxylic acid
groups. Heparin is found within mast cell granules and is released upon
25 degranulation. A cell associated form of heparin is termed heparan sulfate.
Heparan sulfate is a broad term used to describe a variety of sulfated
proteoglycans (HSPG's) found with a near-ubiquitous distribution on
mammalian cell surface membranes and in the extracellular matrix. HSPG
contains a variable percentage of pentameric heparin-like sequences that
30 function in a similar fashion as soluble heparin. The HSPG's serve as a

repository for antithrombin III (ATIII) and for heparin-binding growth factors such as fibroblast growth factors (FGF) 1-5, IL-8, GM-CSF and IL-3.

Folkman et al., *Inflammation: Basic Principles and Clinical Correlates*, 2d Ed. Chapter 40, pp 821-839 (1992). In fact, cells made genetically deficient in HSPG's require exogenous heparin for growth.

Heparin is commonly administered in doses of up to 400 U/kg during surgical procedures such as cardiopulmonary bypass, cardiac catheterization and hemodialysis procedures in order to prevent blood coagulation during such procedures. The anticoagulant effect of heparin in blood is a result of the interaction of heparin with ATIII. The heparin/ATIII complex is a potent inhibitor of many of the clotting factors of the coagulation cascade. Specific inhibition has been demonstrated for activated Factors IXa, Xa, XIa, XIIIa and thrombin. The heparin/ATIII complex has the highest affinity for Factor Xa and thrombin which are common to both the intrinsic and extrinsic clotting pathways involved as the last two steps of the clotting cascade that results in the conversion of fibrinogen to fibrin.

When heparin is administered for anticoagulant effects during surgery, it is an important aspect of post-surgical therapy that the effects of heparin are promptly neutralized so that normal coagulation function can be restored. Currently protamine is used to neutralize heparin. Protamines are simple proteins of low molecular weight which are commonly isolated from salmon sperm. They are rich in arginine amino acid residues and strongly basic. Administered alone, protamines (usually in the form of protamine sulfate) have anti-coagulant effects. When administered in the presence of heparin, a stable complex is formed and the anticoagulant activity of both drugs is lost. Significant hypotensive and anaphylactoid effects of protamine have limited its clinical utility.

Other reported compounds which have heparin neutralizing activity include platelet factor 4 (PF4) and major basic protein, see U.S. Patent

No. 5,086,164. Major basic protein demonstrates heparin neutralizing activity but is also highly toxic.

Angiogenesis

Angiogenesis is closely associated with endothelial cell proliferation and constitutes the development of new capillary blood vessels. As such, it is an important process in mammalian development and growth, and in menstruation processes. The release of angiogenic growth factors, such as fibroblast growth factors 1-5, induces proliferation of endothelial cells via a heparin-dependent receptor binding mechanism. See Yayon et al., *Cell*, 64:841-848 (1991). These heparin-binding growth factors can be released due to vascular trauma (wound healing), immune stimuli (autoimmune disease), inflammatory mediators (prostaglandins) and from tumor cells.

Angiogenesis is also associated with a number of pathological conditions in which it would be desirable to inhibit such new blood vessel development. As one example, angiogenesis is critical to the growth, proliferation, and metastasis of various tumors. Other pathological conditions associated with angiogenesis include diabetic retinopathy, retrolental fibroplasia, neovascular glaucoma, psoriasis, angiofibromas, immune and non-immune inflammation including rheumatoid arthritis, capillary proliferation within atherosclerotic plaques, hemangiomas, endometriosis and Kaposi's Sarcoma.

Folkman et al., *supra*, discloses that psoriatic lesions in the skin are dominated by epithelial proliferation, neovascularization, and an infiltrate of inflammatory cells. It is unclear, however, whether angiogenesis is a step in the pathogenesis of psoriasis or a secondary phenomenon.

Several substances are known to function as angiogenesis inhibitors and have been reported to inhibit tumor angiogenesis, to prevent the onset of arthritis and to inhibit established arthritis in collagen-induced arthritis models, Peacock et al., *J. Exp. Med.*, 175, 1135-1138 (1992). As one example, protamine is known to inhibit tumor angiogenesis and subsequent

tumor growth. According to Taylor et al., *Nature*, 297:307-312 (1982) protamine's anti-angiogenic activity is attributed to its ability to bind heparin. PF4 is also known to exhibit anti-angiogenic activity. Of interest to the present application is U.S. Patent No. 5,112,946 which discloses modified PF4 and analogs thereof which have anti-angiogenic activity but lack the ability to bind heparin. PF4 has been shown to have at least two functional properties. Heparin binding has been studied most extensively; however, PF4 was originally described to have collagenase inhibitory properties. Collagenase inhibitors were the first inhibitors of angiogenesis to be discovered. See Folkman, 1973, *supra*. The mutations in the heparin binding region of PF4 were not examined for their effect on collagenase inhibitory activity. Interestingly, thrombospondin is also an inhibitor of angiogenesis and binds to heparin with a serine/tryptophan motif instead of a basic amino acid motif. Thus, there is no obvious single consensus sequence heparin binding or for angiogenesis inhibition.

Published PCT patent application WO 92/01003 discloses the use of glycosaminoglycan (heparin) derivatives and their use as inhibitors of tumor invasiveness. Heparin derivatives are disclosed which are described as being substantially devoid of anticoagulation activity and which impede the formation of tumor metastases in a host.

Chronic Inflammation

Chronic inflammation is usually accompanied by angiogenesis. Arthritis is a chronic syndrome characterized by the inflammation of the peripheral joints accompanied by synovial thickening and the influx of immune factors and cells such as polymorphonuclear leukocytes (PMN). In rheumatoid arthritis, the inflammation is immune driven, while in reactive arthritis, inflammation is associated with infection of the synovial tissue with pyogenic bacteria or other infectious agents. Folkman et al., 1973, *supra*, also note that many types of arthritis progress from a stage dominated by an inflammatory infiltrate in the joint to a later stage in which a neovascular pannus invades the

joint and begins to destroy cartilage. While it is unclear whether angiogenesis in arthritis is a causative component of the disease, and not an epiphenomenon, there is evidence that angiogenesis is necessary for the maintenance of synovitis in rheumatoid arthritis. While nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids and other therapies have provided improvements in relief for treatment of arthritis, there remains a need in the art for more effective therapies for arthritis and other inflammatory diseases.

Inflammation and angiogenesis are now understood to be separable but not mutually exclusive processes. Specific angiogenic proteins have been discovered that stimulate angiogenesis without inflammation whereas angiostatic steroids can inhibit angiogenesis without decreasing acute inflammation. See Folkman, 1973, *supra*. Interestingly, endotoxin has been identified as the most potent exogenous stimulator of angiogenesis through its stimulation of macrophage cytokines and growth factors.

Bactericidal/Permeability-Increasing Protein

Bactericidal/permeability-increasing protein (BPI) is a protein isolated from the granules of mammalian PMNs, which are blood cells essential in the defense against invading microorganisms. Human BPI protein has been isolated from polymorphonuclear neutrophils by acid extraction combined with either ion exchange chromatography [Elsbach, *J. Biol. Chem.*, 254:11000 (1979)] or *E. coli* affinity chromatography [Weiss, et al., *Blood*, 69:652 (1987)], referred to herein as natural BPI, and has potent bactericidal activity against a broad spectrum of gram-negative bacteria. The molecular weight of human BPI is approximately 55,000 daltons (55kD). The amino acid sequence of the entire human BPI protein, as well as the DNA encoding the protein, have been elucidated in Figure 1 of Gray et al., *J. Biol. Chem.*, 264:9505 (1989), incorporated herein by reference.

The bactericidal effect of BPI has been shown to be highly specific to sensitive gram-negative species, while non-toxic for other microorganisms and for eukaryotic cells. The precise mechanism by which

BPI kills bacteria is as yet unknown, but it is known that BPI must first attach to the surface of susceptible gram-negative bacteria. This initial binding of BPI to the bacteria involves electrostatic interactions between the basic BPI protein and the negatively charged sites on lipopolysaccharides (LPS). LPS has been referred to as "endotoxin" because of the potent inflammatory response that it stimulates. LPS induces the release of mediators by host inflammatory cells which may ultimately result in irreversible endotoxic shock. BPI binds to lipid A, the most toxic and most biologically active component of LPS.

In susceptible bacteria, BPI binding is thought to disrupt LPS structure, leading to activation of bacterial enzymes that degrade phospholipids and peptidoglycans, altering the permeability of the cell's outer membrane, and initiating events that ultimately lead to cell death. Elsbach and Weiss, *Inflammation: Basic Principles and Clinical Correlates*, eds. Gallin et al., Chapter 30, Review Press, Ltd. (1992). BPI is thought to act in two stages. The first is a sublethal stage that is characterized by immediate growth arrest, permeabilization of the outer membrane and selective activation of bacterial enzymes that hydrolyze phospholipids and peptidoglycan. Bacteria at this stage can be rescued by plating on serum albumin supplemented media. The second stage, defined by growth inhibition that cannot be reversed by serum albumin, occurs after prolonged exposure of the bacteria to BPI and is characterized by extensive physiologic and structural changes, including penetration of the cytoplasmic membrane.

BPI is also capable of neutralizing the endotoxic properties of LPS to which it binds. Because of its gram-negative bactericidal properties and its ability to neutralize LPS, BPI can be utilized for the treatment of mammals suffering from diseases caused by gram-negative bacteria, such as bacteremia or sepsis.

A proteolytic fragment corresponding to the N-terminal portion of human BPI holoprotein possesses the lipid A binding and antibacterial

activity of the naturally-derived 55 kD human holoprotein. In contrast to the N-terminal portion, the C-terminal region of the isolated human BPI protein displays only slightly detectable anti-bacterial activity. Ooi, et al., *J. Exp. Med.*, 174:649 (1991). A BPI N-terminal fragment, comprising approximately the first 199 amino acid residues of the human BPI holoprotein and referred to as "rBPI₂₃", has been produced by recombinant means as a 23 kD protein. Gazzano-Santoro et al., *Infect. Immun.* 60:4754-4761 (1992).

Of interest to the present application are the disclosures in PCT International Application PCT/US91/05758 having publication No. WO 92/03535 relating to compositions comprising a BPI protein and an anionic compound which compositions are said to exhibit (1) no bactericidal activity and (2) endotoxin neutralizing activity. Anionic compounds are preferably a protein such as serum albumin but can also be a proteoglycan such as heparin. In addition, Weiss et al., *J. Clin. Invest.*, 55:33-42 (1975) discloses that heparin sulfate and LPS bind to block expression of the permeability increasing activity of BPI. Neither reference discloses neutralization of heparin by combination with BPI, however.

There continues to exist a need in the art for new products and methods for use in neutralization of heparin, inhibition of tumor and angiogenesis, endothelial cell proliferation and treatment of chronic inflammation.

SUMMARY OF THE INVENTION

According to one aspect of the invention, methods are provided for neutralizing the anti-coagulant activity of heparin comprising administering an effective amount of a BPI protein product *in vivo* to a subject or *in vitro* to a fluid sample containing heparin.

According to another aspect of the invention, a BPI protein product is administered to subjects in order to inhibit endothelial cell proliferation including but not limited to endothelial cell proliferation

associated with angiogenesis. The invention provides methods of inhibiting angiogenesis associated with a variety of clinical conditions. Specifically provided by the invention are methods of treating cancer by inhibiting angiogenesis associated with malignant tumor proliferation; Kaposi's sarcoma lesions and the like. Cancers susceptible to treatment by administration of BPI protein products include melanoma, sarcomas, and carcinomas including but not limited to breast, colon, lung, and prostate carcinomas. Other conditions for which BPI protein products can be administered for inhibition of angiogenesis include ocular retinopathy, retrolental fibroplasia, psoriasis, angiofibromas, endometriosis, hemangiomas and the like. Also contemplated by the invention are methods of contraception comprising delivering of an effective amount of a BPI protein product so as to prevent implantation of a fertilized ovum.

The invention also provides methods of treating chronic inflammatory disease states such as arthritis, psoriasis, inflammatory bowel disease, Crohn's disease, ulcerative colitis, lupus erythematosus, autoimmune uveitis, Lyme disease, and asthma comprising administering an effective amount of a BPI protein product to a subject suffering from the inflammatory disease state.

The invention also provides methods of preparation of medicaments for neutralization of the anti-coagulant properties of heparin, inhibition of tumor and endothelial cell proliferation, inhibition of angiogenesis and treatment of chronic inflammatory disease states.

Such medicaments can be prepared for oral administration for injection or other parenteral methods and preferably include conventional pharmaceutically acceptable carriers and adjuvants as would be known to those of skill in the art. The medicaments are preferably in the form of a unit dose in solid, semi-solid and liquid dosage forms such as tablets, pills, powders, liquid solutions or suspensions, and injectable and infusible solutions.

Effective dosage ranges from about 100 $\mu\text{g/kg}$ to about 10 mg/kg of body weight are contemplated.

As used herein, "BPI protein product" includes naturally and recombinantly produced bactericidal/permeability-increasing protein; natural,
5 synthetic, and recombinant biologically active polypeptide fragments of bactericidal/permeability increasing protein; and biologically active polypeptides or analogs, including hybrid fusion proteins, of either bactericidal/permeability increasing protein or biologically active fragments thereof.

10

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 depicts a graph of a heparin binding assay for rBPI_{23} and rBPI ;

15 Fig. 2 depicts a graph showing the effect of heparin on rBPI_{23} binding to *E. coli* J5 lipid A compared to various LPS and teichoic acid samples;

Fig. 3 depicts a graph showing the effect of rBPI_{23} on ATIII/heparin inhibition of thrombin;

20 Fig. 4 depicts a graph showing the effect of rBPI_{23} on ATIII/heparin inhibition of Factor Xa;

Fig. 5 depicts a graph showing the effect of rBPI_{23} on heparin-mediated lengthening of thrombin time in human plasma;

Fig. 6 depicts a graph showing the effect of rBPI_{23} on partial thromboplastin time;

25 Fig. 7 depicts a graph showing the effect of rBPI_{23} and thaumatin control protein on arthritic scores in a collagen-induced arthritis model with mild arthritis;

30 Fig. 8 depicts a graph showing the effect of rBPI_{23} and protamine on arthritic scores in a collagen-induced arthritis model with severe arthritis;

Fig. 9 depicts a graph showing the effect of rBPI₂₃ on the incidence of arthritis in a *Yersinia*-induced arthritis model;

Fig. 10 depicts a graph showing the effect of rBPI₂₃ on inhibition of *Borrelia burgdorferi* LPS-like stimulation of the LAL assay;

5 Fig. 11 depicts a graph showing survival of mice treated with BPI or a buffer in a mouse melanoma metastasis model;

Fig. 12 depicts a graph showing the effect of rBPI₂₃ on Type II murine capillary endothelial cell proliferation;

Fig. 13 illustrates BPI binding to epithelial cells;

10 Fig. 14 depicts a graph of a heparin binding assay for synthetic BPI peptides;

Fig. 15 depicts a graph of a *Limulus* Amoebocyte Lysate (LAL) inhibition assay for synthetic BPI peptides;

15 Fig. 16 depicts a graph of a radial diffusion bactericidal assay for synthetic BPI peptides;

Fig. 17 depicts a graph showing the effect of synthetic BPI peptides in a heparin binding assay;

Figs. 18a and 18b depict graphs showing the effect of synthetic BPI peptides on ATIII/heparin inhibition of thrombin;

20 Figs. 19a and 19b depict graphs showing the effect of synthetic BPI peptides in an LAL inhibition assay;

Figs. 20a, 20b, 20c, and 20d depict graphs showing the effect of synthetic BPI peptides in radial diffusion bactericidal assays;

25 Figs. 20e and 20f depict graphs showing the effect of synthetic BPI peptides in *E. coli* broth assays;

Figs. 21a and 21b depict HPLC absorbance results for proteolytic fragments of rBPI₂₃;

Fig. 22 depicts a graph of LAL inhibition assay results for proteolytic fragments of rBPI₂₃; and

30 Fig. 23 depicts the functional domains of rBPI₂₃.

DETAILED DESCRIPTION

The present invention relates to the administration of bactericidal/permeability-increasing protein (BPI) protein products for the treatment of a variety of therapeutic conditions not directly associated with bacterial infection.

While BPI protein products as described herein are useful as potent cytotoxins for gram-negative bacteria and for neutralizing the adverse effects of lipopolysaccharide associated with the cell walls of gram-negative bacteria, a variety of therapeutic effects for BPI protein products not directly associated with the gram-negative bacterial infection have been discovered. Specifically, the invention provides methods for treating conditions not directly associated with gram-negative infections including neutralization of the anti-coagulant activity of heparin, inhibition of tumor and endothelial cell proliferation including cell proliferation associated with angiogenesis and treatment of chronic inflammatory disease states such as arthritis.

The BPI protein products including biologically active fragments of BPI holoprotein which are to be administered according to the methods of this invention may be generated and/or isolated by any means known in the art. Co-owned, copending U.S. Patent Application Ser. No. 07/885,501, and a continuation-in-part thereof, U.S. Patent Application Ser. No. 08/072,063 filed May 19, 1993 which are both hereby incorporated by reference, disclose novel methods for the purification of recombinant BPI protein products expressed in and secreted from genetically transformed mammalian host cells in culture and discloses how one may produce large quantities of recombinant BPI products suitable for incorporation into stable, homogeneous pharmaceutical preparations.

Biologically active fragments of BPI include biologically active molecules that contains the same amino acid sequence as a BPI holoprotein, except that the molecule lacks amino-terminal amino acids, internal amino acids, and/or carboxy-terminal amino acids of the holoprotein. By way of

nonlimiting examples, such fragments include those described herein and the previously mentioned natural 25 kD fragment and a recombinant 23 kD, 199 amino acid residue amino-terminal fragment of the human BPI holoprotein referred to as rBPI₂₃. See, Gazzano-Santoro et al., *Infect. Immun.* 60:4754-4761 (1992). In that publication, an expression vector was used as a source of DNA encoding a recombinant expression product (rBPI₂₃) having the 31-residue signal sequence and the first 199 amino acids of the N-terminus of the mature human BPI, as set out in SEQ ID NOs: 1 and 2 taken from Gray et al., *supra*, except that valine at position 151 is specified by GTG rather than GTC and residue 185 is glutamic acid (specified by GAG) rather than lysine (specified by AAG). Recombinant holoprotein referred to herein as rBPI or rBPI₅₀ has also been produced having the sequence set out in SEQ ID NOs: 1 and 2 taken from Gray et al., *supra*, with the exceptions noted for rBPI₂₃.

Other non-limiting examples of biologically active fragments of BPI include fragments of, *e.g.*, the BPI holoprotein or of rBPI₂₃ generated upon subjecting the proteins to chemical cleavage with agents such as cyanogen bromide (CNBr) or enzymatic digestion with agents such as endoproteinase Asp-N. BPI protein fragments may also be provided in the form of linear or cyclic synthetic peptides comprising replicas of from about 5 to about 50 continuous amino acids within the BPI holoprotein and especially within the amino terminal half of the protein. Such peptides may be provided in monomeric form, in the form of dimers or multimers (where the peptide replicates a region of BPI having cysteine residues) and in the form of "linear" dimers or multimers wherein a BPI sequence is present repeatedly in the peptide, with or without separation by "spacer" amino acids allowing for selected conformational presentation.

Biologically active analogs of BPI include but are not limited to recombinant hybrid fusion proteins comprising BPI holoprotein or biologically active fragment thereof, and at least a portion of at least one other polypeptide. Such proteins are described by Theofan et al. in co-owned,

compending U.S. Patent Application Ser. No. 07/885,911, and a continuation-in-part application thereof, U.S. Patent Application Ser. No. 08/064,693 filed May 19, 1993 [Attorney Docket No. 27129/31429], which are incorporated herein by reference in their entirety and include hybrid fusion proteins
5 comprising, at the amino terminal end, a BPI protein or a biologically active fragment thereof and, at the carboxy terminal end, at least one constant domain of an immunoglobulin heavy chain or allelic variant thereof.

Biologically active analogs of BPI also include but are not limited to BPI protein products wherein one or more amino acid residues has
10 been replaced by a different amino acid or by atypical amino acids. For example, co-owned, compending U.S. Patent Application Ser. No. 08/013,801 (Theofan et al., "Stable Bactericidal/Permeability-Increasing Protein Products and Pharmaceutical Compositions Containing the Same"), filed February 2, 1993, which is incorporated herein by reference, discloses polypeptide analogs
15 of BPI and BPI fragments wherein a cysteine residue at position 132 or at position 135 is replaced by a different amino acid. One preferred protein product designated rBPI_{23Δ} cys comprises the first 199 amino acid residues of BPI holoprotein but wherein the cysteine residue at position 132 is substituted with an alanine.

20 The administration of BPI protein products is preferably accomplished with a pharmaceutical composition comprising a BPI protein product and a pharmaceutically acceptable diluent, adjuvant, or carrier. The BPI protein product composition may be administered without or in conjunction with known antibiotics, surfactants, or other chemotherapeutic
25 agents. A preferred pharmaceutical composition containing BPI protein products comprises BPI at a concentration of 1 mg/ml in citrate buffered saline (0.02 M citrate, 0.15 M NaCl, pH 5.0) comprising 0.1 % by weight of poloxamer 188 (Pluronic F-68, BASF Wyandotte, Parsippany, NJ) and 0.002 % by weight of polysorbate 80 (Tween 80, ICI Americas Inc.,
30 Wilmington, DE). Such preferred combinations are described in co-owned,

compending, U.S. Patent Application Ser. No.08/012,360 (McGregor et al., "Improved Pharmaceutical Composition"), filed February 2, 1993, the disclosure of which is incorporated herein by reference.

5 Effective doses of BPI and BPI protein products for partial or complete neutralization of the anti-coagulant activity of heparin and other effects described herein may be readily determined by those of skill in the art according to conventional parameters including the size of the subject, the quantity of heparin administered to the subject and the time since administration of the heparin.

10 Other aspects and advantages of the present invention will be understood upon consideration of the following illustrate examples. Example 1 addresses assay systems for quantification of heparin binding by BPI protein products; Example 2 describes the relative capacity of heparin to block binding of bacterial LPS to BPI protein products; Examples 3 and 4, respectively,
15 present results of tests for the capacity of BPI protein products to inhibit thrombin or Factor Xa inactivation by antithrombin III/heparin complexes; and Example 5 relates to the effect of BPI protein products on heparin-mediated lengthening of thrombin time. Example 6 relates to the effect of BPI protein products on heparin mediated lengthening of partial thromboplastin time.
20 Examples 7-9 relate to administration of BPI protein products in model systems of in collagen and bacterial induced arthritis animal model systems exemplifying treatment of chronic inflammatory disease states. Examples 10-11 illustrate testing of BPI protein products for angiostatic effects in a mouse malignant melanoma metastasis model system. Example 12 addresses effects
25 of BPI protein products on endothelial cell proliferation and possible binding mechanisms involved. Example 13 relates to preparation of synthetic BPI peptides. Examples 14-16 illustrate heparin-binding, LPS-binding and bactericidal activities for the synthetic BPI peptides of Example 13. Example 17 relates to preparation of additional synthetic BPI peptides. Examples 18
30 through 21 address properties of the peptides of Example 17. Example 22

discloses the preparation of BPI proteolytic fragment peptides. Example 23 discloses the bactericidal effects of the BPI proteolytic fragments. Example 24 discloses the heparin binding properties of the BPI proteolytic fragments. Example 25 discloses the effect of BPI proteolytic fragments on an LAL assay.

5

Example 1

HEPARIN BINDING BY BPI PROTEIN PRODUCTS

Heparin binding assays were conducted using membrane bound natural and recombinant BPI molecules and radiolabelled heparin. Briefly, rBPI₂₃ and holoprotein designated rBPI or rBPI₅₀ were added to wells of a 96-well microtiter plate having an Imobilon-P (Millipore, Bedford, MA) membrane disposed at the bottom of the wells. Five μ g of protein was added to each well. The wells were dried and subsequently blocked with a 0.1 % bovine serum albumin (BSA) in phosphate buffered saline, pH 7.4 (blocking buffer.) Dilutions of ³H-heparin (DuPont, NEN, Wilmington, DE) were made in the blocking buffer and incubated in the BPI containing wells for one hour at 4°C. The unbound heparin is aspirated and the wells were washed three times with blocking buffer, dried and removed for quantitation in a liquid scintillation counter. Typical assay results are graphically presented in Figure 1. While BSA in the blocking buffer does have a low affinity and capacity to bind heparin, this was considered physiologically irrelevant and the background was routinely subtracted from the test compound signal. The binding of radiolabeled heparin was completely inhibited by a 100 fold excess of unlabeled heparin (data not shown).

Similar assays compared heparin binding by rBPI₂₃, rBPI₅₀, and natural holoprotein (BPI) with thaumatin control protein (having charge and size similar to rBPI₂₃) or with a wash buffer (1 % BSA) control. In these assays, less heparin binding by the natural and recombinant BPI holoproteins was observed. The lesser extent of binding by rBPI and nBPI may have been the result of carbohydrate contamination of the protein preparations.

In addition, binding constants with ^3H -heparin as the ligand were determined using nonlinear function minimization with Grafit software (Erithicus Softward Ltd., Staines, UK) for rBPI₂₃, rBPI, protamine sulfate (Sigma Chemical Co.) and thaumatin with the results shown in Table 1 below.

TABLE 1

Binding Constants with ^3H -heparin as the Ligand

PROTEIN	K _d	CAPACITY	HEPARIN COLUMN NaCl ELUTION
rBPI ₂₃	79 nM	2.63 μg	0.84 M
rBPI ₅₀	173 nM	1.30 μg	0.81 M
Protamine	8.1 nM	2.66 μg	1.33 M
Thaumat	no binding		0.15 M

Example 2

HEPARIN COMPETITION FOR BPI PROTEIN PRODUCT BINDING

The ability of heparin, soluble lipid A, LPS, and Teichoic acids to compete with immobilized *E. coli* J5 lipid A for binding to soluble rBPI₂₃ was assessed. Specifically, Immulon 2 (Dynatech, Chintilly, VA) microtiter wells were coated with *E. coli* J5 lipid A at a concentration of 0.5 $\mu\text{g}/\text{mL}$ in methanol (50 μL volume). The wells were then blocked for 4 hours at 37°C with PBS containing 0.1% BSA. Control wells were treated with 50 μL of plain methanol and then blocked as above. The blocked wells were aspirated and washed twice with PBS/0.05 % Tween-20. Varying concentrations of putative inhibitors were plated onto the wells in a volume of 25 μL PBS, followed by 200,000 cpm of radio-iodinated rBPI₂₃ in 25 μL of PBS containing 0.1% Tween-20. The test solutions included Ra LPS from *Salmonella minnesota* R60 at 200 $\mu\text{g}/\text{mL}$; smooth LPS from *E. coli* 0113 (RIBI Immunochem, Hamilton, MT, #R318) at 200 $\mu\text{g}/\text{mL}$; lipoteichoic acid from

Streptococcus faecalis, (Sigma, St. Louis, MO, #L-4015) at 400 $\mu\text{g/mL}$;
lipoteichoic acid from *Staphylococcus Aureus*, (Sigma #L-2525) at 400 $\mu\text{g/mL}$;
and heparin sodium USP injection (Lypho-Med, Rosemont, IL, #9155-01) at
400 $\mu\text{g/mL}$. Binding was allowed to proceed overnight at 4°C with gentle
shaking, after which the wells were aspirated, washed three times with
PBS/0.05 % Tween-20, and counted. The results as set out in Figure 2 show a
high affinity of rBPI₂₃ for heparin and also that heparin blocks BPI binding to
lipid A.

Example 3

HEPARIN NEUTRALIZATION BY BPI PROTEIN PRODUCTS: EFFECT OF BPI ON THROMBIN INACTIVATION BY ATIII/HEPARIN COMPLEXES

A chromogenic assay was used to determine the effect of rBPI₂₃
on thrombin inactivation by ATIII/heparin complexes. Specifically, a
Chromostate™ anti-thrombin assay kit (Organon Teknika Corp., Durham, NC)
was used to examine the inhibition of purified thrombin by preformed
ATIII/heparin complexes in plasma.

The assay was performed in 96 well microtiter plates in
triplicate with a final volume per well of 200 μL . The order of addition of
assay components was as follows: 1) 50 μl sample (rBPI₂₃ or thaumatin as a
control protein) with final concentrations of 100, 50, 25, 10 and 1 $\mu\text{g/well}$ in
PBS; 2) 50 μl plasma 1:100 in buffer; 3) 50 μl thrombin at 1 nKat/mL in
buffer; and 4) 50 μl chromogenic substrate 1 $\mu\text{mol/mL}$ in H₂O. The reaction
was allowed to proceed for 10 minutes at 37°C and stopped with 50 μL 0.1M
citric acid and the color reaction was quantitated on a microplate reader. The
assay results shown in Figure 3 indicate that rBPI₂₃ can effectively neutralize
ATIII/heparin complexes in heparinized human plasma in a dose dependent
manner. As the plasma was titrated the amount of thrombin activity increased.
This was caused by a decrease in the amount of inhibitory ATIII/heparin
complexes in the added plasma. The control protein, thaumatin, showed no

similar neutralizing effect and was essentially equivalent to the buffer control at all protein concentrations.

Example 4

5 **HEPARIN NEUTRALIZATION BY BPI PROTEIN PRODUCTS: EFFECT OF BPI ON FACTOR Xa INACTIVATION BY ATIII/HEPARIN COMPLEXES**

10 A chromogenic assay was used to determine the effect of rBPI₂₃ on Factor Xa neutralization by ATIII/heparin complexes. Specifically, the assay was conducted using a chromostrate heparin anti-Factor Xa assay kit (Organon Teknika Corp.) and was performed under fixed concentrations of Factor Xa and ATIII. Heparin concentration was varied so that a heparin standard curve was generated for heparin at concentrations of 1, 0.5, 0.25, 0.125, 0.063, 0.031, 0.016, 0.008, 0.004, 0.002, and 0 units/mL in PBS. The assay measured functional Factor Xa activity by the release of a chromogenic compound from a synthetic substrate. ATIII/heparin complexes neutralize Factor Xa activity, thus the amount of chromogen released was inversely related to the amount and anti-Factor Xa activity of heparin in the assay sample.

20 The assay was performed in 96 well microtiter plates in triplicate with a final volume per well of 200 μ L. Assay components were added to the microtiter wells as follows: 1) 50 μ L samples (rBPI₂₃ or thaumatin as a control protein) with final concentrations of 100, 50, 25, 10 and 1 μ g/well in PBS; 2) 50 μ L Factor Xa 0.14nKat.mL in H₂O; 3) 25 μ L ATIII at 0.5 U/mL in H₂O; 4) 24 μ L heparin at 0.25 U/mL in buffer; 5) 50 μ L substrate (3 μ moles/mL). The reaction was allowed to proceed for 10 minutes at 37°C and stopped with 50 μ l of 0.1M citric acid and then the color reaction was quantitated on a microplate reader. The assay results shown in Figure 4 indicates that rBPI₂₃ can effectively neutralize heparin in the ATIII/heparin inhibition of Factor Xa. As the concentration of heparin is increased, the amount of rBPI₂₃ necessary for heparin neutralization also

increased. The control protein, thaumatin, showed no similar neutralizing effects and was essentially equivalent to the buffer control at all protein concentrations.

Example 5

HEPARIN NEUTRALIZATION BY BPI PROTEIN PRODUCTS: EFFECT OF BPI ON HEPARIN-MEDIATED LENGTHENING OF THROMBIN TIME

The effect of BPI protein products on heparin-mediated lengthening of thrombin time, i.e., the time required for clotting of a mixture of thrombin and plasma was examined. Thrombin time is lengthened by the presence of endogenous or exogenous inhibitors of thrombin formation, such as therapeutically administered heparin. Agents which neutralize the anti-coagulant effects of heparin will reduce the thrombin time measured by the test. Human citrated plasma (200 μ L) was incubated for 1 minute at 37°C with either 15 μ L of diluent (0.15 M NaCl, 0.1 M Tris, pH 7.4) or 15 μ L of the diluent also containing 25 μ g/mL heparin (187 units/mg). Various concentrations of rBPI₂₃ (from 0.0 to 56 μ g/mL) in a volume of 15 μ L were added, followed immediately by 100 μ L of thrombin reagent (Sigma Chemical Co., No. 845-4). Clotting time (thrombin time) was measured using a BBL Fibrometer (Becton Dickenson Microbiology Systems, Cockeysville, MD). The results shown in Figure 5 establish that rBPI₂₃ inhibits the heparin-mediated lengthening of thrombin time. In the absence of heparin, rBPI₂₃ had no effect on the assay even at concentrations as high as 56 μ g/mL.

Example 6

HEPARIN NEUTRALIZATION BY BPI PROTEIN PRODUCTS: EFFECT OF BPI ON PARTIAL THROMBOPLASTIN TIME

The effect of rBPI₂₃, or protamine sulfate on partial thromboplastin time (PTT) in heparinized rats was determined. PTT is lengthened by the presence of endogenous or exogenous inhibitors of thrombin

formation, such as therapeutically administered heparin. Agents which neutralize the anti-coagulant effects of heparin will reduce the PTT as measured by the test. Sprague-Dawley rats housed under NIH guidelines were administered with 100 U/kg heparin by bolus intravenous injections via the animals' tail vein followed by administration of 5 mg/kg rBPI₂₃, 5 mg/kg protamine sulfate or buffer. The PTT was then determined from blood samples collected from the abdominal aorta of the previously anesthetized animals. The PTT of untreated animals was also determined. The results shown in Figure 6 establish that both rBPI₂₃ and protamine had an immediate effect on the PTT of the treated animals. These animal data confirm the heparin neutralizing effects of BPI protein products as shown in Examples 2-5.

The collective results from Examples 1 through 6 show that rBPI₂₃ binds to heparin in direct binding assays and effectively neutralizes heparin inhibition of coagulation proteases. Based on these characteristics, BPI protein products are projected to be useful in the clinical neutralization of heparin anti-coagulant effects in dosages generally corresponding functionally to those recommended for protamine sulfate, but are not expected to possess the severe hypotensive and anaphylactoid effects of that material.

20

Example 7

THERAPEUTIC EFFECTS OF BPI PROTEIN PRODUCTS FOR CHRONIC INFLAMMATORY DISEASE: COLLAGEN INDUCED ARTHRITIS MODEL

A further aspect of the present invention relates to the discovery of the utility of BPI to treat and prevent the effects of chronic inflammatory disease states such as arthritis, including rheumatoid and reactive arthritis, psoriasis, inflammatory bowel disease, Crohn's disease, ulcerative colitis, lupus erythematosus, autoimmune uveitis, Lyme disease, and asthma. Exemplary methods are provided for treating subjects suffering from arthritis comprising administering an effective amount of a BPI protein product in order

30

to prevent or treat arthritis. The BPI protein product may be administered topically, or by injection such as intraarticularly, intravenously, intramuscularly or subcutaneously or by other parenteral and non-parenteral methods.

5 The effect of administration of BPI protein products was studied in a collagen-induced arthritis model. Specifically, arthritis was induced in mice by intradermal immunization of bovine Type II collagen at the base of the tail according to the method of Stuart et al., *J. Clin. Invest.*, 69:673-683 (1982). Generally, mice begin to develop arthritic symptoms at Day 21 after
10 collagen immunization. The arthritic scores of the treated mice were then evaluated in a blinded fashion over a period of 120 days for mice treated on each of days 21-25 with doses of either rBPI₂₃, thaumatin control protein, or buffer which were injected intravenously via the tail vein.

 Specifically, bovine Type II collagen (Southern Biotechnology Associates, Inc., Birmingham AL) was administered via intradermal injection
15 (0.1 mg/mouse) at the base of the tail on day 0 to groups of ten male mice (Mouse/DBA/1J), each weighing approximately 20-25 g. rBPI₂₃ was dissolved in 0.5 M NaCl, 20 mM sodium acetate, pH 6.0 and diluted with PBS buffer (1 mg/ml) for administration at 0.125 mg/mouse. Thaumatin protein in PBS
20 (0.121 mg/mouse) or PBS buffer alone (0.1 ml) were administered as controls.

 The results shown in Figure 7 demonstrate that the rBPI₂₃ has a statistically significant effect in reducing the arthritic score of treated mice compared with the PBS buffer and thaumatin protein controls.

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Example 8

THERAPEUTIC EFFECTS OF BPI PROTEIN PRODUCTS IN COMPARISON WITH PROTAMINE SULFATE FOR CHRONIC INFLAMMATORY DISEASE: COLLAGEN INDUCED ARTHRITIS MODEL

 The collagen-induced arthritis model of Example 7 was used to
30 evaluate the performance of a BPI protein product in comparison with

protamine sulfate, using both thaumatin protein and buffer as controls. Specifically, rBPI₂₃ was dissolved in 0.5 M NaCl, 20 mM sodium acetate, pH 6.0 and diluted with PBS buffer (1 mg/ml) and was administered at 0.125 mg/mouse. The other test materials were administered at the following dosages: protamine sulfate (Sigma Chemical Co) (0.13 mg/mouse), thaumatin (0.121 mg/mouse), and PBS buffer (0.1 ml). Each of four groups of ten mice received test or control materials through intravenous injection via the tail vein on each of days 28 through 32. Figure 8 discloses the results of arthritic scores for the various treatment and control protocols evaluated at days 28-80. The stars (*) in Fig. 8 represent a statistically significant difference between rBPI₂₃ and buffer at $p < 0.01$ while the pluses (+) represent a statistically significant difference between rBPI₂₃ and buffer at $p < 0.05$. These results show that the rBPI₂₃ significantly reduced arthritic score for mice treated in the model system.

Example 9

GRAM-NEGATIVE INDUCED REACTIVE ARTHRITIS MODELS

The effect of administration of BPI protein products to treat reactive arthritis was studied in a *Yersinia enterocolitica* reactive arthritis model according to the method of Yong et al., *Microbial Pathogenesis*, 4:305-310 (1988). Specifically, BPI protein products are administered to DBA/2J male mice which had previously been injected intravenously through the tail vein with *Yersinia enterocolitica* cWA 0:8 T2 (i.e., lacking the virulence plasmid according to Yong et al. *supra*) at a dosage of 4×10^8 bacteria calculated to induce a non-septic arthritis in the mice. Each of three groups of 15 mice each received test or control materials through intravenous injection via the tail vein. The mice were given either rBPI₂₃ at a dosage of about 5.0 mg/kg dissolved in a buffer of 20mM sodium citrate, 150 mM sodium chloride, 0.1 % poloxamer 188, 0.002 % polysorbate 80, pH 5.0; thaumatin protein at a dosage of about 5.0 mg/kg dissolved in the buffer of 20mM

sodium citrate, 150 mM sodium chloride, 0.1 % poloxamer 188, 0.002 % polysorbate 80, pH 5.0; or the buffer alone. The results depicted in Fig. 9 show that the BPI protein product significantly reduced the incidence of reactive arthritis versus the buffer or thaumatin control protein.

5 *Borrelia burgdorferi* is the pathogen responsible for Lyme Disease and associated arthritis and it possesses an LPS-like complex on its cell walls which is different from but structurally related to that of *E. coli*. The effect of administration of BPI protein products on inhibition of *Borrelia burgdorferi* LPS in a *Limulus* Amoebocyte Lysate (LAL) inhibition assay was
10 determined. Specifically, an LAL assay according to the method of Example 15 was conducted measuring the effect of rBPI₂₃ on *Borrelia burgdorferi* LPS administered at 2.5 µg/mL and *E. coli* 0113 LPS administered at 2 ng/mL. The results depicted in Fig. 10 show that rBPI₂₃ neutralizes the effects of both *Borrelia burgdorferi* LPS and *E. coli* 0113 LPS in the LAL assay.

15

Example 10

EFFECT OF BPI IN A MOUSE MALIGNANT MELANOMA MODEL

According to this example, a BPI protein product, protamine, and both thaumatin protein and buffer controls were tested for efficacy in a
20 mouse malignant melanoma metastasis model. Specifically, four groups of nine C57BL/6J mice were inoculated with 10⁵ B16.F10 malignant melanoma cells via intravenous injection into the tail vein on day 0. Either rBPI₂₃ (0.13 mg/mouse), protamine sulfate (0.13 mg/mouse), thaumatin (0.13 mg/mouse) or PBS buffer (0.1 ml/mouse) were intravenously administered into the tail
25 vein of the mice on days 1, 3, 6, 8, 10, 13, 15, 17, and 19. The animals were sacrificed via cervical dislocation on Day 20 for observation of lung tissues. The lobes of each lung were perfused and inflated by injecting 3 ml water into the lung via the trachea. Superficial tumor nodules were then counted with the aid of a dissecting microscope and the number of tumors
30 found per group analyzed for statistically significant differences. While the

data was not statistically significant, animals treated with BPI₂₃ had the lowest tumor load, followed by those treated with protamine, the thaumatin protein control and the buffer control. The lack of statistical significance (tumor number did not adequately reflect tumor size) indicated that a more specific assay methodology would be needed to determine the tumor load.

Example 11

EFFECT OF BPI IN A MOUSE MALIGNANT MELANOMA MODEL

A BPI protein product, protamine, and both thaumatin protein and buffer controls were again tested for efficacy in the mouse malignant melanoma metastasis model of Example 10. Specifically, six groups of C57BL/6J mice were inoculated with 10⁵ B16.F10 malignant melanoma cells via intravenous injection into the tail vein on day 0. Either rBPI₂₃ (0.125 mg/mouse), protamine sulfate (0.125 mg/mouse), thaumatin (0.125 mg/mouse) or PBS buffer as set out in Table 2 below were intravenously administered into the tail vein of the mice on days 1, 2, 5, 7, 9, 12, 14, 16, and 19. All animals in groups A-D were sacrificed by cervical dislocation on day 20 for observation of lung tissues. The lungs were removed and placed into a beaker of cold water. The lobes of each lung were then perfused and inflated by injecting 3 ml of water into the lung via the trachea. Superficial tumor nodules are then analyzed for melanin content.

TABLE 2

Group	Control/Test Article	No. of Animals
A	Buffer	10
B	Protamine	10
C	Thaumatin	10
D	rBPI ₂₃	10
E	Buffer	5
F	rBPI ₂₃	5

Groups E and F comprising animals treated with either buffer or rBPI₂₃ respectively were not sacrificed but were observed once daily for mortality. Figure 11 shows the survival data for the two groups of animals. Although all ten of the animals had died by day 43, the BPI treated mice generally survived significantly longer than the untreated mice indicating that BPI had an anti-angiogenic effect and slowed metastasis of the melanoma tumors.

Given the above, according to an additional aspect of the invention, BPI protein products may be used to inhibit Kaposi's Sarcoma in a model system such as that of Miles et al., VII International Conference on AIDS, Florence, Italy, Paper 41(8), 1991.

Example 12

EFFECT OF BPI ON ENDOTHELIAL CELL PROLIFERATION

Murine cerebral capillary endothelial cells (EC) as described in Bauer, *Microvascular Research* 37:148-161 (1989) were passaged in Medium 199 containing Earle's salts, L-glutamine and 2.2 g/l of sodium bicarbonate (Gibco, Grand Island, NY, #400-100EB), plus 10% heat inactivated fetal calf serum (Irvine Scientific, Irvine, CA) and 1% penicillin/streptomycin (Gibco, #600-5140AG). Harvesting of the confluent cells was performed by trypsinization with trypsin-EDTA (Gibco #610-5300PG) for 3 minutes. The trypsinization was stopped by adding 10 ml of the passage medium to the flask. Proliferation assays were performed on freshly harvested EC in standard flat bottom 96 well microtiter plates. A final volume of 200 μ l/well was maintained for each well of the assay. A total of 4×10^4 EC were added to each well with varying concentrations of rBPI₂₃, thaumatin control protein or buffer control. After 48 hours of culture in a 5% CO₂ incubator, 1 μ Ci of [methyl-³H] thymidine in 10 μ l of Medium 199 was added to each well. After a 24 hour pulse, the EC cells were harvested by trypsinization onto glass

microfiber filters and incorporated [^3H]thymidine was quantitated with a gas proportional solid phase beta counter.

Concentration dependent inhibition of EC cell proliferation by rBPI_{23} is shown in Figure 12. No effect was observed when similar concentrations of thaumatin or equal volumes of the buffer were added to the wells. The first inhibition of proliferation is observed at $12.4 \mu\text{g/ml}$ rBPI_{23} and the effect appears to be maximal at $50 \mu\text{g/ml}$. The growth of the EC cells is known to be dependent on FGF-2 (bFGF) in the calf serum and FGF-2 requires cell surface heparan for receptor activation (Yayon et al., *Cell* 64:841-848, 1991). Without intending to be bound by a theory of the invention, it is believed that rBPI_{23} bound to cell surface heparan on the EC cells interferes with the activation of the cells by FGF-2.

Direct binding studies of rBPI_{23} on the EC cells were performed by harvesting the 10x passaged cells from a confluent flask and resuspending the trypsinized cells in 12.5 ml of culture medium. 0.5 ml of the suspension was added to each well of a standard 24 well tissue culture plate and incubated overnight. The plate was washed with 0.1 % bovine serum albumin in phosphate buffered saline containing calcium and magnesium. (Gibco.) After washing, 0.5 ml of the BSA/PBS was added per well. Preliminary experiments indicated that 50 ng/ml of ^{125}I -labeled rBPI_{23} added to the wells produced approximately 30,000 specific cpm after a 3 hour, 4°C incubation with 3x washing in PBS and lysis with 1M NaOH from gamma counting of the lysate.

The specific binding of 50 ng/ml ^{125}I -labeled, "hot" rBPI_{23} to the EC cells could be competed by addition of $20 \mu\text{g/ml}$ heparin (Sigma, Grade I). Similar competition was observed for unlabeled ("cold") rBPI_{23} added to the binding culture. The combination of unlabeled rBPI_{23} with heparin (concurrently added or pre-mixed prior to addition) could not reduce the binding below the heparin only competition (Figure 13). These data indicate that rBPI_{23} binds to endothelial cells via heparin-like molecule and that this

binding appears to interfere with EC cell proliferation to a heparin binding growth factor (FGF-2).

Example 13

5 PREPARATION OF 15-MER SYNTHETIC PEPTIDES OF BPI

In order to assess biological properties of peptide fragment BPI protein products, 15-mer amino acid synthetic peptides based on the 23 kD amino terminal fragment of BPI were prepared and evaluated for heparin-binding activity, activity in a *Limulus* Amoebocyte Lysate Inhibition (LAL) assay and bactericidal activity. Specifically, 47 synthetic peptides each
10 comprising 15 amino acids and overlapping the adjacent peptides by 11 amino acids were prepared, in duplicate, based on the sequence of rBPI₂₃ described above.

Peptides were simultaneously synthesized according to the methods of Maeji et al., *Immunol. Methods*, 134:23-33 (1990) and Gammon et al., *J. Exp. Med.*, 173:609-617 (1991), utilizing the solid-phase technology of Cambridge Research Biochemicals Ltd. under license of Coselco Mimotopes Pty Ltd. Briefly, the sequence of rBPI₂₃ (1-199) was divided into 47 different
15 15-mer peptides that progressed along the linear sequence of rBPI₂₃ by initiating a subsequent peptide every fifth amino acid. This peptide synthesis technology allows for the simultaneous small scale synthesis of multiple peptides on separate pins in a 96-well plate format. Thus, 94 individual pins were utilized for this synthesis and the remaining to pins (B,B) were subjected to the same steps as the other pins without the addition of activated FMOC-amino acids. Final cleavage of the 15-mer peptides from the solid-phase pin
20 support employed an aqueous basic buffer (sodium carbonate, pH 8.3). The unique linkage to the pin undergoes a quantitative diketopiperazine cyclization under these conditions resulting in a cleaved peptide with a cyclo(lysylprolyl) moiety on the carboxyl-terminus of each peptide. The amino-termini were not
25 acetylated so that the free amino group could potentially contribute to anion
30

binding reactions. An average of about 15 μg of each 15-mer peptide is recovered per well.

Example 14

5 HEPARIN BINDING BY 15-MER SYNTHETIC PEPTIDES OF BPI

The synthetic BPI protein product peptides described above were subjected to a heparin binding assay according to the methods described in Example 1. The results, as shown in Figure 14, indicate the existence of three separate functional domains with heparin binding activity; the first extending
10 from about amino acids 21-55; the second extending from about amino acids 65-107; and the third extending from about amino acids 137-171. Material from blank control pins had no heparin binding effects.

Example 15

15 EFFECT OF 15-MER SYNTHETIC PEPTIDES OF BPI ON AN LAL ASSAY

The synthetic BPI protein product peptides were subjected to a *Limulus* Amoebocyte Lysate (LAL) inhibition assay to determine LPS binding properties. Specifically, the synthetic BPI peptides were mixed in Eppendorf
20 tubes with a fixed concentration of *E. coli* 0113 LPS (4 ng/ml final) and incubated at 37°C for 3 hours with occasional shaking. Addition controls comprising 0.05 $\mu\text{g}/\text{mL}$ were also tested. Following incubation, 360 μl D-PBS was added per tube to obtain an LPS concentration of 200 pg/mL for the LAL assay. Each sample was then transferred into Immulon II strips
25 (Dynatech, Chantilly, VA) in volumes of 50 μl per well.

Limulus amoebocyte Lysate (Quantitative chromogenic LAL kit, Whitaker Bioproducts, Inc., Walkersville, MD) was added at 50 μl per well and the wells were incubated at room temperature for 25 minutes. Chromogenic substrate was then added at a volume of 100 μl per well and was
30 well mixed. After incubation for 20 to 30 minutes at room temperature, the reaction was stopped with addition of 100 μl of 25% acetic acid. Optical

density at 405 nm was then measured in a multiplate reader (Vmax, Molecular Dynamics, Menlo Park, CA) with the results shown in Figure 15 in terms of percent inhibition of LPS. The data in Figure 15 indicate at least three major domains with significant LAL inhibition; the first extending from amino acids 17-55; the second extending from about amino acids 73-99 and the third extending from about amino acids 137-163. Other individual peptides also exhibit LAL inhibition. In contrast, material from blank control pins did not exhibit LPS neutralizing effects as measured by the LAL assay.

10

Example 16

BACTERICIDAL EFFECTS OF 15-MER SYNTHETIC PEPTIDES OF BPI.

The synthetic BPI protein product peptides were tested for bactericidal effects against the rough mutant *E. coli* J5 bacteria in a radial diffusion assay. Specifically, an overnight culture of *E. coli* J5 was diluted 1:50 into fresh tryptic soy broth and incubated for 3 hours at 37°C to attain log phase. Bacteria were then pelleted at 3,000 rpm for 5 minutes in a Sorvall RT6000B. 5mL of 10 mM sodium phosphate buffer (pH 7.4) was added and the preparation was re-centrifuged. The supernatant was decanted and 5 mL of fresh buffer was added, the bacteria were resuspended and their concentration was determined by measurement of absorbance at 590 nm. Adsorbance of 1.25×10^9 CFU/mL suspension equals 1.00. The bacteria were diluted to 4×10^6 CFU/mL in 10 mL of molten underlayer agarose (approximately 45°C) and inverted repeatedly to mix with 15 mL polypropylene tubes used for this purpose.

25

The entire contents of the tube were poured into a perfectly level square petri dish and distributed evenly by rocking the dish side to side. The agarose hardened in less than 30 seconds and had a uniform thickness of about 1 mm. A series of wells were then punched into the hardened agarose using a sterile 3 mm punch attached to a vacuum apparatus. The punch was sterilized with 100% alcohol and allowed to air dry.

30

10 μL of the synthetic BPI peptides were carefully pipetted into each well. As controls, pH 8.3 buffer was added to a separate well (as positive controls, 5 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$ concentration of rBPI₂₃ was also added. In addition, products from the blank pins B and B were tested as
5 controls. The plate was allowed to incubate at 37°C for 3 hours and 10 mL of molten overlayer agarose (at approximately 45°C) was then added into the level petri dish, allowed to harden and incubated overnight at 37°C. A clear zone was seen against the lawn of bacteria in those wells having bactericidal activity. In order to visually enhance this zone, a dilute Coomassie solution
10 (0.002 % Coomassie Brilliant Blue, 27 % methanol, 15 % formaldehyde (37 % stock solution) and H₂O) was poured over the agar and allowed to stain for 24 hours. The bacterial zones were measured with a Mitutoyo micrometer.

The results of the assay are shown in Figure 16 where the only synthetic BPI peptide seen to have bactericidal activity was a fragment
15 corresponding to amino acids 85-99. The positive rBPI₂₃ controls also had bactericidal effects while the buffer and blank pin controls did not.

Example 17

PREPARATION OF BPI PEPTIDE FRAGMENTS

20 Based on the results of testing of overlapping peptides in Examples 13 through 16, BPI protein product peptide fragments were prepared by solid phase peptide synthesis according to the methods of Merrifield, *J. Am. Chem. Soc.* 85: 2149 (1963) and Merrifield et al., *Anal. Chem.* 38: 1905-1914 (1966) using an Applied Biosystems, Inc. Model 432 synthesizer. Nine
25 BPI protein product peptides designated BPI-2 through BPI-10 were prepared having the amino acid sequences of portions of amino acid residues 1-199 of rBPI₂₃ as set out in Table 3 below. In the cases of BPI-7, BPI-9 and BPI-10 the peptides represented partial or even multiple repeats of sequence. Specifically, BPI-7 comprises a 20-mer consisting of amino acid residues 90-
30 99 repeated twice in a single linear chain. BPI-10 comprises a 30-mer

consisting of amino acid residues 90-99 repeated three times in a single linear chain. BPI-9 comprises a 16-mer comprising amino acid residues 94-99 followed by residues 90-99 in a single linear chain.

TABLE 3

5	BPI Protein Product Peptides			
	Polypeptide No.	Amino Acid Region	Amino Acid Residues	MW (daltons)
	BPI-2	85-99	15	1828.16
	BPI-3	73-99	27	3072.77
10	BPI-4	25-46	22	2696.51
	BPI-5	142-163	22	2621.52
	BPI-6	112-127	16	1569.82
	BPI-7	90-99, 90-99	20	2644.66
	BPI-8	90-99	10	1316.8
15	BPI-9	94-99, 90-99	16	2131.34
	BPI-10	90-99, 90-99, 90,99	30	3958.45

Example 1820 **HEPARIN BINDING BY BPI PROTEIN PRODUCT PEPTIDES**

In this example BPI protein product peptides BPI-2, BPI-3, BPI-4, BPI-6, BPI-7, and BPI-8 along with BPI cys were subjected to a heparin binding assay according to the methods described in Example 1. The results, as shown in Fig. 17 indicate that BPI-7, and BPI-8 have extremely high heparin binding capacity while BPI-2 and BPI-3 have more moderate heparin binding capacity and BPI-4 and BPI-6 have little or no heparin binding capacity.

Example 19**HEPARIN NEUTRALIZATION BY BPI PROTEIN PRODUCT PEPTIDES**

In this example BPI protein product peptides BPI-2, BPI-3, BPI-4, BPI-5, BPI-6, BPI-7, and BPI-8 along with rBPI₂₃ were subjected to an assay to determine their effect on thrombin inactivation by ATIII/heparin complexes according to the method of Example 3. Varying concentrations of the BPI protein products ranging from 1.0 µg/mL to 100 µg/mL were administered to determine their effect. BPI protein peptides BPI-7, BPI-3, and BPI-5 each had the most significant heparin neutralization effects as shown in Figs 18a and 18b which depict the sample concentrations as weight or molar concentrations respectively.

Example 20**EFFECT OF BPI PROTEIN PRODUCT PEPTIDES ON AN LAL ASSAY**

In this example BPI protein product peptides BPI-2, BPI-3, BPI-4, BPI-6, BPI-7, and BPI-8 along with rBPI₂₃ were subjected to an LAL assay according to the method of Example 15 to determine their LPS binding and inhibition properties. The results show that BPI-7 and BPI-3 had significant LPS inhibition properties, that BPI-2 and BPI-8 had moderate LPS inhibition properties and that BPI-4 and BPI-6 had no significant LPS inhibition activity as depicted in Figs. 19a and 19b which depict the sample concentrations as weight or molar concentrations respectively.

Example 21**BPI PROTEIN PRODUCT PEPTIDE BACTERICIDAL ASSAY**

In this example, BPI protein product peptides BPI-2, BPI-3, BPI-4, BPI-5, BPI-6, BPI-7, BPI-8, BPI-9 and BPI-10 along with rBPI₂₃ were tested for bactericidal effects against mutant *E. coli* J5 (rough) and *E. coli* 0111:B4 (smooth) bacteria in a radial diffusion assay according to the methods of Example 16. The results depicted in Figs. 20a-20d show that each of BPI-

2, BPI-3, BPI-5, BPI-7, BPI-8, BPI-9 and BPI-10 have greater or lesser degrees of bactericidal activity while BPI-4 and BPI-6 exhibited no bactericidal activity. The bactericidal peptides each tended to be more effective against the rough than the smooth *E. coli* strain.

As a further aspect of this example, broth antibacterial assays were conducted to determine the bactericidal activity of certain of the BPI peptides. Specifically either *E. coli* J5 (rough) and *E. coli* 0111:B4 (smooth) bacteria were selected from single colonies on agar plates and used to inoculate culture plates to which were added serial ten-fold dilutions of the BPI protein product peptides. The plates were incubated overnight and read on an ELISA plate reader to determine the surviving colony forming units. The results of this assay are depicted in Figs. 20e (*E. coli* J5) and 20f (*E. coli* 0111:B4) which show that BPI protein product peptides BPI-3, BPI-7, BPI-9 and BPI-10 have significant anti-bacterial activity.

The results of these bactericidal assays along with the heparin binding and LAL assays indicate that there exist small synthetic BPI peptides with one or more of bactericidal, heparin binding and LPS neutralizing effects and that there exist at least three distinct separate functional domains within the 23 kD amino terminal fragment. One domain resides between amino acid residues 17 and 45. A second, the most active domain, characterized by activity in all three assays, resides between amino acids 71 and 99. One specific peptide 86-99 demonstrated activity in all three assays. A third domain is composed of residues 142-169.

25 Example 22

PREPARATION OF BPI PROTEOLYTIC FRAGMENT PEPTIDES

In this example chemical cleavage and enzymatic digestion processes were applied to rBPI₂₃, produced according to the procedures of Gazzano-Santoro *et al.*, *supra*, to develop variously sized proteolytic fragments of the recombinant protein.

The rBPI₂₃ was reduced and alkylated prior to proteolysis by cyanogen bromide (CNBr) or endoproteinase Asp-N. The protein was desalted by cold (4°C) acetone precipitation (1:1 v/v) overnight and pelleted by centrifugation (5000 xg) for 10 minutes. The rBPI₂₃ pellet was washed twice with cold acetone and dried under a stream of nitrogen. The rBPI₂₃ was then reconstituted to 1 mg/ml in 8M urea/0.1M Tris, pH 8.1 and reduced by addition of 3.4 mM dithiothreitol (Calbiochem, San Diego, CA) for 90 minutes at 37°C. Alkylation was performed by the addition of iodoacetamide (Sigma Chemical Co., St. Louis, MO) to a final concentration of 5.3 millimolar for 30 minutes in the dark at room temperature. The reduced and alkylated protein was acetone precipitated, centrifuged and washed as described above and the pellet was redissolved for either CNBr or Asp-N digestion.

Prior to CNBr addition, the washed pellet was dissolved in 70 % trifluoroacetic acid (TFA) (protein sequencing grade, Sigma) to a final protein concentration of 5 mg/ml. Cyanogen bromide (Baker Analyzed Reagent, VWR Scientific, San Francisco, CA) dissolved in 70 % TFA was added to give a final 2:1 ratio of CNBr to protein (w/w). This is approximately a 75 fold molar excess of CNBr over methionine residues in the protein. The reaction was purged with nitrogen and allowed to proceed for 24 hours in the dark at room temperature. The reaction was terminated by adding 9 volumes of distilled water, and followed by freezing (-70°C) and lyophilization.

The reduced and alkylated rBPI₂₃ was solubilized at 5.0 mg/ml in 8M urea/0.1M Tris, pH 8.1. An equal volume of 0.1M Tris, pH 8.1 was added so that the final conditions were 2.5 mg/ml protein in 5M urea/0.1M Tris, pH 8.1. Endoproteinase Asp-N from *Pseudomonas fragi* (Boehringer-Mannheim, Indianapolis, IN) was added at a 1:1000 (w/w) enzyme:substrate ratio and the digest was allowed to proceed for 6 hours at 37°C. The reaction was terminated by addition of TFA to a final concentration of 0.1 % and the samples were then fractionated by reverse phase HPLC.

The CNBr and Asp-N fragment mixtures were purified on a Zorbax Protein Plus C₃ column (4.6 x 250 mm, 300 Å pore size, MACMOD Analytical Inc, Chadsford, PA). A gradient from 5% acetonitrile in 0.1% TFA to 80% acetonitrile in 0.1% TFA was run over 2 hours at 1.0 ml/min. Fragment elution was monitored at 220 nm using a Beckman System Gold HPLC. The column heating compartment was maintained at 35°C and the fractions were collected manually, frozen at -70°C and dried in a Speed Vac concentrator. Fragments were then solubilized in 20 mM sodium acetate, pH 4.0/0.5 M NaCl prior to use.

Electrospray ionization mass spectrometry was performed on a VG Bio-Q mass spectrometer by Dr. Francis Bitsch and Mr. John Kim in the laboratory of Dr. Cedric Shackleton, Children's Hospital-Oakland Research Institute. Molecular masses were obtained by mathematical transformation of the data.

Although the DNA sequence for rBPI₂₃ encodes amino acid residues 1-199 of the mature protein, a significant portion of the protein that is produced that is produced is truncated at Leu-193 and Val 195 as determined by electrospray ionization mass spectrometry (ESI-MS). These C-terminal truncations were verified by isolating the C-terminal tryptic peptides, which were sequenced and analysed by ESI-MS. There are six methionine residues, at positions 56, 70, 100, 111, 170, and 196 and chemical cleavage by cyanogen bromide produced six major peptide fragments as predicted. The results of the CNBr cleavage experiments are summarized in Table 4. The fragments were isolated by reverse phase (C₃) HPLC (Figure 21A) and their N-terminal sequences were determined by Edman degradation. The two largest fragments (C1 and C5) were not resolved by the C₃ HPLC column and further attempts to resolve them by ion exchange chromatography were unsuccessful, presumably because they are similar in length and isoelectric point. The identities of the C1, C5 fragments within the mixture were determined by ESI-MS. The predicted mass of C1 is 6269 (Table 4), taking

into account the loss of 30 a.m.u. resulting from the conversion of the C-terminal methionine to homoserine during the CNBr cleavage reaction. The observed mass of 6251.51 ± 0.34 is consistent with the loss of a water molecule (18 a.m.u.) in a homoserine lactone intermediate, which may be
5 favored over the formation of the homoserine because of the hydrophobicity of the C1 fragment C-terminal amino acids. The predicted mass of the C5 fragment is 6487 and the observed mass is 6385.84 ± 0.39 (Table 1). For the C5 fragment, the C-terminal amino acids are hydrophilic, so the hydrolysis of the homoserine lactone intermediate is probably favored. From both the N-
10 terminal sequencing and the mass spectrum data, the C5 component represents approximately 10-25% of the material in the C1/C5 mixture.

Proteolytic cleavage with endoproteinase Asp-N was performed to provide additional fragments for the regions contained within the CNBr C1/C5 mixture. There are six aspartic acid residues within the rBPI₂₃
15 sequence at positions 15, 36, 39, 57, 105, and 116. The six major Asp-N fragments isolated by C₃ HPLC (Figure 21B) were sequenced and masses were determined by ESI-MS (Table 4). A short duration digest at a 1:1000 (w/w) enzyme:substrate ratio was used to eliminate potential non-specific cleavages, particularly at glutamic acid. It is evident that this digestion did not continue
20 until completion, as one fragment (1-38) was isolated where Asp residues (amino acids 15 and 35) were not cleaved. The mass spectra of the Asp-N fragments were consistent with the predicted masses for each individual fragment. Unlike the CNBr cleavage, where the C-terminal fragment was poorly resolved, the Asp-N fragment from amino acid 116 to the C-terminus
25 was well resolved from all of the other Asp-N fragments.

TABLE 4**SUMMARY OF rBPI₂₃ CLEAVAGE FRAGMENT ANALYSIS****CNBr Cleavage Fragments**

	<u>PEAK</u>	<u>SEQUENCE</u>	<u>I.D.</u>	<u>MASS</u>	
				<u>measured</u>	<u>predicted</u>
5	I	101-110	C4(101-111)	Not Determined	1169
	II	57-67	C2(57-70)	Not Determined	1651
	III	71-99	C3(71-100)	Not Determined	3404
	IV	171-194	C6(171-196)	Not Determined	2929
10	V	1-25, 112-124	C1(1-56), C5(112-170)	6251.51 \pm 0.34 6485.84 \pm 0.39	6299 6403

Asp-N Proteolytic Fragments

	<u>PEAK</u>	<u>SEQUENCE</u>	<u>I.D.</u>	<u>MASS</u>	
				<u>measured</u>	<u>predicted</u>
15	A	1-14	A1(1-14)	1465.5	1464
	I	39-56	A3(39-56)	2145.2	2145
	II	15-38	A2(15-38)	2723.6	2724
	III	57-76	A4(57-104)	5442.5	5442
20	IV	1-38	A1 A2(1-38)	4171.4	4172
	VI	116-134	A6a(116-193)	8800.3	8800
	VII	116-128	A6b(116-195)	8997.1	8996

Example 23

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BACTERICIDAL EFFECTS OF BPI PROTEOLYTIC FRAGMENTS

BPI proteolytic fragments developed according to Example 22 were screened for bactericidal effects rough mutant *E. coli* J5 bacteria in a radial diffusion assay essentially according to the procedures of Example 16. No bactericidal activity was demonstrated for the rBPI₂₃ fragments generated by CNBr or by Asp-N digestion, when tested up to 25 pmol/well. This assay

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detected measurable bactericidal activity with as little as 0.75 pmol of rBPI₂₃ per well. Reduced and alkylated rBPI₂₃ (up to 100 pmol/well) also was not bactericidal, while alkylated rBPI₂₃ retained bactericidal activity equivalent to rBPI₂₃.

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Example 24

HEPARIN BINDING BY BPI PROTEOLYTIC FRAGMENTS

rBPI₂₃ and BPI proteolytic fragments developed according to Example 22 were employed in heparin binding assays essentially according to the procedures of Example 1.

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Heparin binding of CNBr fragments was estimated using 100 picomoles of each fragment per well with a saturating concentration of ³H-heparin (20 µg/ml). The results as shown in Table 5 (means of duplicate wells plus or minus the range between the two valves) indicate that the CNBr fragments containing the amino acids 71-100 (C3) and 1-56 and 112-170 (C1,5) bound heparin to a similar extent. The CNBr fragment 171-193 also bound more heparin than the control protein, thaumatin, a protein of similar molecular weight and charge to rBPI₂₃.

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The Asp-N fragments also demonstrated multiple heparin binding regions in rBPI₂₃. As seen in Table 5, the 57-104 Asp-N fragment bound the highest amount of heparin, followed by the 1-38 and 116-193 fragments. These data, in combination with the CNBr fragment data, indicate that there are at least three separate heparin binding regions within rBPI₂₃, with the highest capacity residing within residues 71-100.

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TABLE 5
Heparin Binding of rBPI₂₃ Fragments

	<u>Fragments</u>	<u>Region</u>	<u>cpm³H-Heparin bound</u>
5	<u>CNBr Digest</u>		
	C1,C5	1-56,112-170	82,918 \pm 4,462
	C2	57-70	6,262 \pm 182
	C3	71-100	81,655 \pm 3,163
	C4	101-111	4,686 \pm 4
10	C6	171-196	26,204 \pm 844
	<u>Asp-N Digest</u>		
	A1	1-38	17,002 \pm 479
	A2	15-38	3,042 \pm 162
15	A3	39-56	8,664 \pm 128
	A4	57-104	33,159 \pm 1,095
	A6a	116-193	13,419 \pm 309
	rBPI ₂₃	1-193	51,222 \pm 1,808
20	Thaumatococcus		7,432 \pm 83
	Wash Buffer		6,366 \pm 46

Example 25

EFFECT OF BPI PROTEOLYTIC FRAGMENTS ON AN LAL ASSAY

25 BPI proteolytic fragments developed according to Example 22 were employed in an LAL inhibition assay essentially as described in Example 15, providing results shown in Figure 22 wherein: the filled triangle represents rBPI₂₃; the open circle represents Asp-N fragment A3; the closed circle represents Asp-N fragment A2; the open square represents Asp-N
30 fragment A3; the filled square represents Asp-N fragment A1A2; the open

triangle represents Asp-N fragment A6b; the small open triangle represents CNBr fragment C3; and the small filled square represents CNBr fragment C1/C5.

5 The CNBr digest fraction containing amino acid fragments 1-56 and 112-170 inhibited the LPS-induced LAL reaction with an IC_{50} of approximately 100 nM. The IC_{50} is approximately 10 fold higher than the IC_{50} for rBPI₂₃ (9 nM) in the same assay. The other CNBr digest fragments were non-inhibitory.

10 A slightly different result was observed with fragments generated from the Asp-N digest, where three fragments were found to be inhibitory in the LAL assay. The fragment corresponding to amino acids 116-193 exhibited LAL inhibitory activity similar to intact rBPI₂₃ with complete inhibition of the LPS-induced LAL reaction at 15 nM. The fragments corresponding to amino acids 57-104 and 1-38 also inhibited the LAL assay, 15 but required 10 fold higher amounts. These results, in combination with the CNBr digest results, indicate that at least three regions of the rBPI₂₃ molecule have the ability to neutralize LPS activation of the LAL reaction with the most potent region appearing to exist within the 116-193 amino acid fragment.

20 In related studies of the proteolytic fragments of Example 22 involving ELISA assays using a rabbit polyclonal anti-rBPI₂₃ antibody capable of blocking rBPI₂₃ bactericidal and LAL inhibition properties and two different, non-blocking mouse anti-rBPI₂₃ monoclonal antibodies, the polyclonal antibody was noted to be immunoreactive with the 116-193 and 57-104 Asp-N fragments as well as the 1-56 and 112-170 CNBr fragments while 25 the murine monoclonal antibodies reacted only with an Asp-N fragment representing residues 1-14 of rBPI₂₃.

Overall, the results indicate that rBPI₂₃ contains three functional domains that contribute to the total biological activity of the molecule. The first domain appears in the sequence of amino acids 17-45 and is destroyed by 30 Asp-N cleavage at residue 36. This domain is moderately active in both the

inhibition of LPS-induced LAL activity and heparin binding assays. The second active domain appears in the region of amino acids 65-99 and its inhibition of LPS-induced LAL activity is diminished by CNBr cleavage at residue 70. This domain also exhibits the highest heparin binding capacity and contains the bactericidal peptide, 85-99. The third active domain, between amino acids 142-169, is active in the inhibition of LPS-induced LAL stimulation assay and exhibits the lowest heparin binding capacity of the three regions.

Other bactericidal proteins, for example, cecropins and magainins, are characterized by a continuous, amphipathic, α -helical region which is necessary for activity. A high degree of structural similarity was observed between the cationic/hydrophobic motif of LPS binding/bactericidal molecules and the consensus sequences of heparin binding proteins. An excellent correlation exists between the synthetic rBPI₂₃ peptides that bind to heparin and those which inhibit the LPS-induced LAL reaction ($r=0.75$, $p=0.0001$, $n=47$) (Figs. 14 through 16). These data suggest that LPS and heparin may present similar charged arrays to the proteins with which they interact. As a result, other proteins which bind to LPS avidly, may also bind tightly to heparin.

Numerous modifications and variations in the practice of the invention are expected to occur to those skilled in the art upon consideration of the presently preferred embodiments thereof. According to one aspect of the invention, methods of treating gram-negative bacterial infections and the sequelae thereof are contemplated which comprise administration of BPI protein product peptides having gram-negative bactericidal activity. Consequently, the only limitations which should be placed upon the scope of the invention are those which appear in the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: KOMA CORPORATION

(ii) TITLE OF INVENTION: Therapeutic uses of
Bactericidal/Permeability-Increasing Protein Products

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Marshall, O'Toole, Gerstein, Murray & Borun
(B) STREET: 6300 Sears Tower, 233 South Wacker Drive
(C) CITY: Chicago
(D) STATE: Illinois
(E) COUNTRY: USA
(F) ZIP: 60606-6402

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY INFORMATION:

(A) NAME: Sharp, Jeffrey S.
(B) REGISTRATION NUMBER: 31,879
(C) REFERENCE/DOCKET NUMBER: 31580

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 312/474-6300
(B) TELEFAX: 312/474-0448
(C) TELEX: 25-3856

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1813 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 31..1491

(ix) FEATURE:

(A) NAME/KEY: mat_peptide
(B) LOCATION: 124..1491

43

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAGGCCTTGA GGTTTTGGCA GCTCTGGAGG ATG AGA GAG AAC ATG GCC AGG GGC	54
Met Arg Glu Asn Met Ala Arg Gly	
-31 -30 -25	
CCT TGC AAC GCG CCG AGA TGG GTG TCC CTG ATG GTG CTC GTC GCC ATA	102
Pro Cys Asn Ala Pro Arg Trp Val Ser Leu Met Val Leu Val Ala Ile	
-20 -15 -10	
GGC ACC GCC GTG ACA GCG GCC GTC AAC CCT GGC GTC GTG GTC AGG ATC	150
Gly Thr Ala Val Thr Ala Ala Val Asn Pro Gly Val Val Val Arg Ile	
-5 1 5	
TCC CAG AAG GGC CTG GAC TAC GCC AGC CAG CAG GGG ACG GCC GCT CTG	198
Ser Gln Lys Gly Leu Asp Tyr Ala Ser Gln Gln Gly Thr Ala Ala Leu	
10 15 20 25	
CAG AAG GAG CTG AAG AGG ATC AAG ATT CCT GAC TAC TCA GAC AGC TTT	246
Gln Lys Glu Leu Lys Arg Ile Lys Ile Pro Asp Tyr Ser Asp Ser Phe	
30 35 40	
AAG ATC AAG CAT CTT GGG AAG GGG CAT TAT AGC TTC TAC AGC ATG GAC	294
Lys Ile Lys His Leu Gly Lys Gly His Tyr Ser Phe Tyr Ser Met Asp	
45 50 55	
ATC CGT GAA TTC CAG CTT CCC AGT TCC CAG ATA AGC ATG GTG CCC AAT	342
Ile Arg Glu Phe Gln Leu Pro Ser Ser Gln Ile Ser Met Val Pro Asn	
60 65 70	
GTG GGC CTT AAG TTC TCC ATC AGC AAC GCC AAT ATC AAG ATC AGC GGC	390
Val Gly Leu Lys Phe Ser Ile Ser Asn Ala Asn Ile Lys Ile Ser Gly	
75 80 85	
AAA TGG AAG GCA CAA AAG AGA TTC TTA AAA ATG AGC GGC AAT TTT GAC	438
Lys Trp Lys Ala Gln Lys Arg Phe Leu Lys Met Ser Gly Asn Phe Asp	
90 95 100 105	
CTG AGC ATA GAA GGC ATG TCC ATT TCG GCT GAT CTG AAG CTG GGC AGT	486
Leu Ser Ile Glu Gly Met Ser Ile Ser Ala Asp Leu Lys Leu Gly Ser	
110 115 120	
AAC CCC ACG TCA GGC AAG CCC ACC ATC ACC TGC TCC AGC TGC AGC AGC	534
Asn Pro Thr Ser Gly Lys Pro Thr Ile Thr Cys Ser Ser Cys Ser Ser	
125 130 135	
CAC ATC AAC AGT GTC CAC GTG CAC ATC TCA AAG AGC AAA GTC GGG TGG	582
His Ile Asn Ser Val His Val His Ile Ser Lys Ser Lys Val Gly Trp	
140 145 150	
CTG ATC CAA CTC TTC CAC AAA AAA ATT GAG TCT GCG CTT CGA AAC AAG	630
Leu Ile Gln Leu Phe His Lys Lys Ile Glu Ser Ala Leu Arg Asn Lys	
155 160 165	
ATG AAC AGC CAG GTC TGC GAG AAA GTG ACC AAT TCT GTA TCC TCC AAG	678
Met Asn Ser Gln Val Cys Glu Lys Val Thr Asn Ser Val Ser Ser Lys	
170 175 180 185	
CTG CAA CCT TAT TTC CAG ACT CTG CCA GTA ATG ACC AAA ATA GAT TCT	726
Leu Gln Pro Tyr Phe Gln Thr Leu Pro Val Met Thr Lys Ile Asp Ser	
190 195 200	
GTG GCT GGA ATC AAC TAT GGT CTG GTG GCA CCT CCA GCA ACC ACG GCT	774
Val Ala Gly Ile Asn Tyr Gly Leu Val Ala Pro Pro Ala Thr Thr Ala	
205 210 215	

44

GAG ACC CTG GAT GTA CAG ATG AAG GGG GAG TTT TAC AGT GAG AAC CAC Glu Thr Leu Asp Val Gln Met Lys Gly Glu Phe Tyr Ser Glu Asn His	822
220 225 230	
CAC AAT CCA CCT CCC TTT GCT CCA CCA GTG ATG GAG TTT CCC GCT GCC His Asn Pro Pro Pro Phe Ala Pro Pro Val Met Glu Phe Pro Ala Ala	870
235 240 245	
CAT GAC CGC ATG GTA TAC CTG GGC CTC TCA GAC TAC TTC TTC AAC ACA His Asp Arg Met Val Tyr Leu Gly Leu Ser Asp Tyr Phe Phe Asn Thr	918
250 255 260 265	
GCC GGG CTT GTA TAC CAA GAG GCT GGG GTC TTG AAG ATG ACC CTT AGA Ala Gly Leu Val Tyr Gln Glu Ala Gly Val Leu Lys Met Thr Leu Arg	966
270 275 280	
GAT GAC ATG ATT CCA AAG GAG TCC AAA TTT CGA CTG ACA ACC AAG TTC Asp Asp Met Ile Pro Lys Glu Ser Lys Phe Arg Leu Thr Thr Lys Phe	1014
285 290 295	
TTT GGA ACC TTC CTA CCT GAG GTG GCC AAG AAG TTT CCC AAC ATG AAG Phe Gly Thr Phe Leu Pro Glu Val Ala Lys Lys Phe Pro Asn Met Lys	1062
300 305 310	
ATA CAG ATC CAT GTC TCA GCC TCC ACC CCG CCA CAC CTG TCT GTG CAG Ile Gln Ile His Val Ser Ala Ser Thr Pro Pro His Leu Ser Val Gln	1110
315 320 325	
CCC ACC GGC CTT ACC TTC TAC CCT GCC GTG GAT GTC CAG GCC TTT GCC Pro Thr Gly Leu Thr Phe Tyr Pro Ala Val Asp Val Gln Ala Phe Ala	1158
330 335 340 345	
GTC CTC CCC AAC TCC TCC CTG GCT TCC CTC TTC CTG ATT GGC ATG CAC Val Leu Pro Asn Ser Ser Leu Ala Ser Leu Phe Leu Ile Gly Met His	1206
350 355 360	
ACA ACT GGT TCC ATG GAG GTC AGC GCC GAG TCC AAC AGG CTT GTT GGA Thr Thr Gly Ser Met Glu Val Ser Ala Glu Ser Asn Arg Leu Val Gly	1254
365 370 375	
GAG CTC AAG CTG GAT AGG CTG CTC CTG GAA CTG AAG CAC TCA AAT ATT Glu Leu Lys Leu Asp Arg Leu Leu Leu Glu Leu Lys His Ser Asn Ile	1302
380 385 390	
GGC CCC TTC CCG GTT GAA TTG CTG CAG GAT ATC ATG AAC TAC ATT GTA Gly Pro Phe Pro Val Glu Leu Leu Gln Asp Ile Met Asn Tyr Ile Val	1350
395 400 405	
CCC ATT CTT GTG CTG CCC AGG GTT AAC GAG AAA CTA CAG AAA GGC TTC Pro Ile Leu Val Leu Pro Arg Val Asn Glu Lys Leu Gln Lys Gly Phe	1398
410 415 420 425	
CCT CTC CCG ACG CCG GCC AGA GTC CAG CTC TAC AAC GTA GTG CTT CAG Pro Leu Pro Thr Pro Ala Arg Val Gln Leu Tyr Asn Val Val Leu Gln	1446
430 435 440	
CCT CAC CAG AAC TTC CTG CTG TTC GGT GCA GAC GTT GTC TAT AAA Pro His Gln Asn Phe Leu Leu Phe Gly Ala Asp Val Val Tyr Lys	1491
445 450 455	
TGAAGGCACC AGGGGTGCCG GGGGCTGTCA GCCGCACCTG TTCCTGATGG GCTGTGGGGC	1551
ACC GGCTGCC TTTCCCCAGG GAATCCTCTC CAGATCTTAA CCAAGAGCCC CTTGCAAAC	1611
TCTTCGACTC AGATTCAGAA ATGATCTAAA CACGAGGAAA CATTATTCAT TGGAAAAGTG	1671

45

CATGGTGTGT ATTTTAGGGA TTATGAGCTT CTTTCAAGGG CTAAGGCTGC AGAGATATTT 1731
 CCTCCAGGAA TCGTGTITTC ATTGTAACCA AGAAATTTCC ATTTGTGCTT CATGAAAAAA 1791
 AACTTCTGGT TTTTTCATG TG 1813

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 487 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Arg Glu Asn Met Ala Arg Gly Pro Cys Asn Ala Pro Arg Trp Val
 -31 -30 -25 -20
 Ser Leu Met Val Leu Val Ala Ile Gly Thr Ala Val Thr Ala Ala Val
 -15 -10 -5 1
 Asn Pro Gly Val Val Val Arg Ile Ser Gln Lys Gly Leu Asp Tyr Ala
 5 10 15
 Ser Gln Gln Gly Thr Ala Ala Leu Gln Lys Glu Leu Lys Arg Ile Lys
 20 25 30
 Ile Pro Asp Tyr Ser Asp Ser Phe Lys Ile Lys His Leu Gly Lys Gly
 35 40 45
 His Tyr Ser Phe Tyr Ser Met Asp Ile Arg Glu Phe Gln Leu Pro Ser
 50 55 60 65
 Ser Gln Ile Ser Met Val Pro Asn Val Gly Leu Lys Phe Ser Ile Ser
 70 75 80
 Asn Ala Asn Ile Lys Ile Ser Gly Lys Trp Lys Ala Gln Lys Arg Phe
 85 90 95
 Leu Lys Met Ser Gly Asn Phe Asp Leu Ser Ile Glu Gly Met Ser Ile
 100 105 110
 Ser Ala Asp Leu Lys Leu Gly Ser Asn Pro Thr Ser Gly Lys Pro Thr
 115 120 125
 Ile Thr Cys Ser Ser Cys Ser Ser His Ile Asn Ser Val His Val His
 130 135 140 145
 Ile Ser Lys Ser Lys Val Gly Trp Leu Ile Gln Leu Phe His Lys Lys
 150 155 160
 Ile Glu Ser Ala Leu Arg Asn Lys Met Asn Ser Gln Val Cys Glu Lys
 165 170 175
 Val Thr Asn Ser Val Ser Ser Lys Leu Gln Pro Tyr Phe Gln Thr Leu
 180 185 190
 Pro Val Met Thr Lys Ile Asp Ser Val Ala Gly Ile Asn Tyr Gly Leu
 195 200 205
 Val Ala Pro Pro Ala Thr Thr Ala Glu Thr Leu Asp Val Gln Met Lys
 210 215 220 225

Gly	Glu	Phe	Tyr	Ser 230	Glu	Asn	His	His 235	Asn	Pro	Pro	Pro	Phe	Ala 240	Pro
Pro	Val	Met	Glu 245	Phe	Pro	Ala	Ala	His 250	Asp	Arg	Met	Val	Tyr 255	Leu	Gly
Leu	Ser	Asp 260	Tyr	Phe	Phe	Asn	Thr 265	Ala	Gly	Leu	Val	Tyr 270	Gln	Glu	Ala
Gly	Val 275	Leu	Lys	Met	Thr	Leu 280	Arg	Asp	Asp	Met	Ile 285	Pro	Lys	Glu	Ser
Lys 290	Phe	Arg	Leu	Thr	Thr 295	Lys	Phe	Phe	Gly	Thr 300	Phe	Leu	Pro	Glu	Val 305
Ala	Lys	Lys	Phe	Pro 310	Asn	Met	Lys	Ile	Gln 315	Ile	His	Val	Ser	Ala 320	Ser
Thr	Pro	Pro	His 325	Leu	Ser	Val	Gln	Pro 330	Thr	Gly	Leu	Thr	Phe 335	Tyr	Pro
Ala	Val	Asp 340	Val	Gln	Ala	Phe	Ala 345	Val	Leu	Pro	Asn	Ser 350	Ser	Leu	Ala
Ser	Leu 355	Phe	Leu	Ile	Gly	Met 360	His	Thr	Thr	Gly	Ser 365	Met	Glu	Val	Ser
Ala 370	Glu	Ser	Asn	Arg	Leu 375	Val	Gly	Glu	Leu	Lys 380	Leu	Asp	Arg	Leu	Leu 385
Leu	Glu	Leu	Lys	His 390	Ser	Asn	Ile	Gly	Pro 395	Phe	Pro	Val	Glu	Leu 400	Leu
Gln	Asp	Ile	Met 405	Asn	Tyr	Ile	Val	Pro 410	Ile	Leu	Val	Leu	Pro 415	Arg	Val
Asn	Glu	Lys 420	Leu	Gln	Lys	Gly	Phe 425	Pro	Leu	Pro	Thr	Pro 430	Ala	Arg	Val
Gln	Leu 435	Tyr	Asn	Val	Val	Leu 440	Gln	Pro	His	Gln	Asn 445	Phe	Leu	Leu	Phe
Gly 450	Ala	Asp	Val	Val	Tyr 455	Lys									

WHAT IS CLAIMED IS:

1. A method for neutralizing the anti-coagulant effect of heparin comprising administering to a subject an effective amount of a heparin binding BPI protein product.
- 5 2. A method of inhibiting angiogenesis comprising administering to a subject an amount of a BPI protein product effective to inhibit angiogenesis.
3. A method for neutralizing the anti-coagulant effect of heparin in a fluid sample comprising contacting said sample with an effective amount of heparin binding BPI protein product.
- 10 4. The method of claim 2 wherein the angiogenesis inhibited is associated with ocular retinopathy.
- 15 5. A method for inhibiting endothelial cell proliferation comprising administering to a subject an amount of a BPI protein product effective to inhibit proliferation.
- 20 6. A method of treating endometriosis comprising administering an amount of BPI to the endometrium effective to inhibit endothelial cell proliferation.
7. A method for contraception comprising administering to a subject an amount of a BPI protein product to the uterine lining effective to prevent endothelial cell proliferation associated with implantation of a fertilized ovum.
- 25 8. A method for inhibiting malignant tumor cell proliferation comprising administering to a subject an amount of a BPI protein product effective to inhibit proliferation.
- 30

9. The method of claim 8 wherein the malignant tumor is Kaposi's sarcoma.

10. A method for treating a chronic inflammatory disease state comprising administering to a subject an amount of a BPI protein product effective to reduce inflammation.

11. The method of claim 10 wherein the chronic inflammatory disease is arthritis.

10

12. The method of claim 11 wherein the arthritic inflammatory disease state is rheumatoid arthritis.

13. The method of claim 11 wherein the arthritic inflammatory disease state treated is reactive arthritis.

15

14. The method of claims 1, 2, 5, 6, 7, 8 and 10 wherein the BPI protein product is a 23-25 kD amino-terminal fragment of bactericidal/permeability-increasing protein.

20

15. A BPI protein product for use as a heparin binding medicament.

16. A BPI protein product for heparin binding use in neutralizing the anti-coagulant effect of heparin.

25

17. A BPI protein product for heparin binding use in inhibiting angiogenesis.

18. A product according to claim 17, wherein the angiogenesis inhibited is associated with ocular retinopathy.

30

19. A BPI protein product for heparin binding use in inhibiting endothelial cell proliferation.

20. A product according to claim 17, wherein the endothelial cell proliferation is associated with endometriosis.

21. A BPI protein product for heparin binding use as a contraceptive.

22. A BPI protein product for heparin binding use in inhibiting malignant tumor cell proliferation.

23. A product according to claim 22, wherein the malignant tumor is Kaposi's sarcoma.

24. A BPI protein product for heparin binding use in treating a chronic inflammatory disease state.

25. A product according to claim 24, wherein the chronic inflammatory disease is arthritis.

26. A product according to claim 25, wherein the arthritic inflammatory disease is rheumatoid arthritis.

27. A product according to claim 25, wherein the arthritic inflammatory disease is reactive arthritis.

28. A product according to any one of claims 15, 16, 17, 19, 21, 22 and 24, which is a 23-25 kD amino-terminal fragment of bactericidal/permeability-increasing protein.

29. Use of a BPI protein product for the manufacture of a heparin binding medicament.

5 30. Use of a BPI protein product for the manufacture of a heparin binding medicament for neutralizing the anti-coagulant effect of heparin.

10 31. Use of a BPI protein product for the manufacture of a heparin binding medicament for inhibiting angiogenesis.

32. Use of a BPI protein product for the manufacture of a heparin binding medicament for inhibiting angiogenesis associated with ocular retinopathy.

15 33. Use of a BPI protein product for the manufacture of a heparin binding medicament for inhibiting endothelial cell proliferation.

20 34. Use of a BPI protein product for the manufacture of a heparin binding medicament for treating endometriosis by inhibiting endothelial cell proliferation.

35. Use of a BPI protein product for the manufacture of a heparin binding contraceptive.

25 36. Use of a BPI protein product for the manufacture of a heparin binding medicament for inhibiting malignant tumor cell proliferation.

30 37. The use according to claim 36, wherein the malignant tumor is Kaposi's sarcoma.

38. Use of a BPI protein product for the manufacture of a heparin binding medicament for treating a chronic inflammatory disease state.

39. The use according to claim 38, wherein the chronic
5 inflammatory disease is arthritis.

40. The use according to claim 39, wherein the arthritic inflammatory disease is rheumatoid arthritis.

10 41. The use according to claim 39, wherein the arthritic inflammatory disease is reactive arthritis.

42. The use according to any one of claims 29, 30, 31, 32, 33, 34, 35, 36 and 38, wherein the protein product is a 23-25 kD amino-
15 terminal fragment of bactericidal/permeability-increasing protein.

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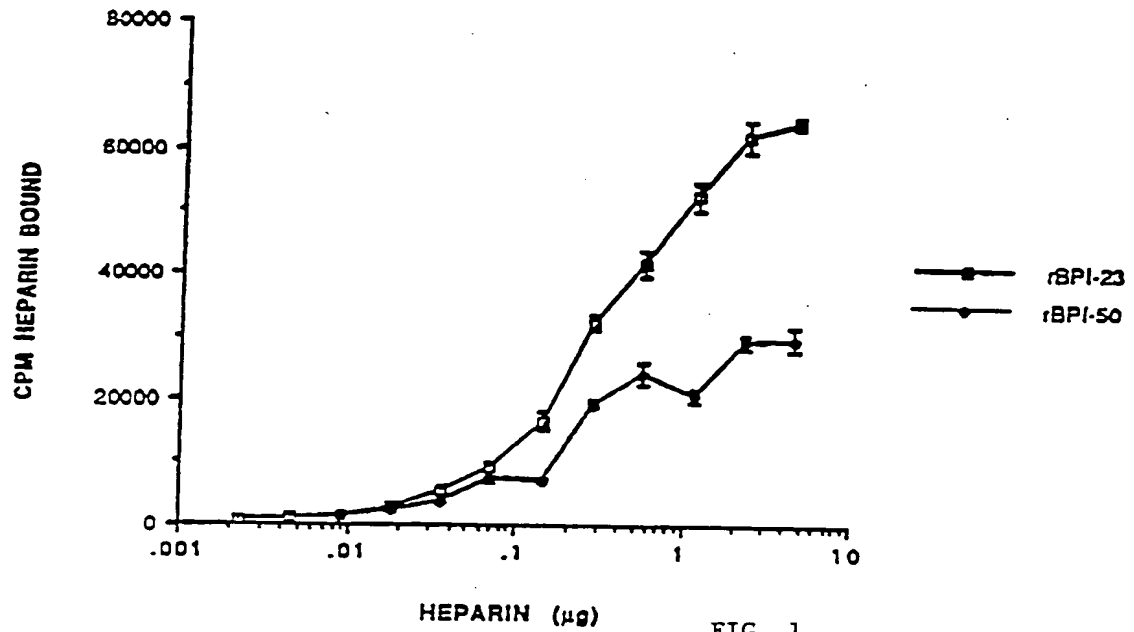


FIG. 1

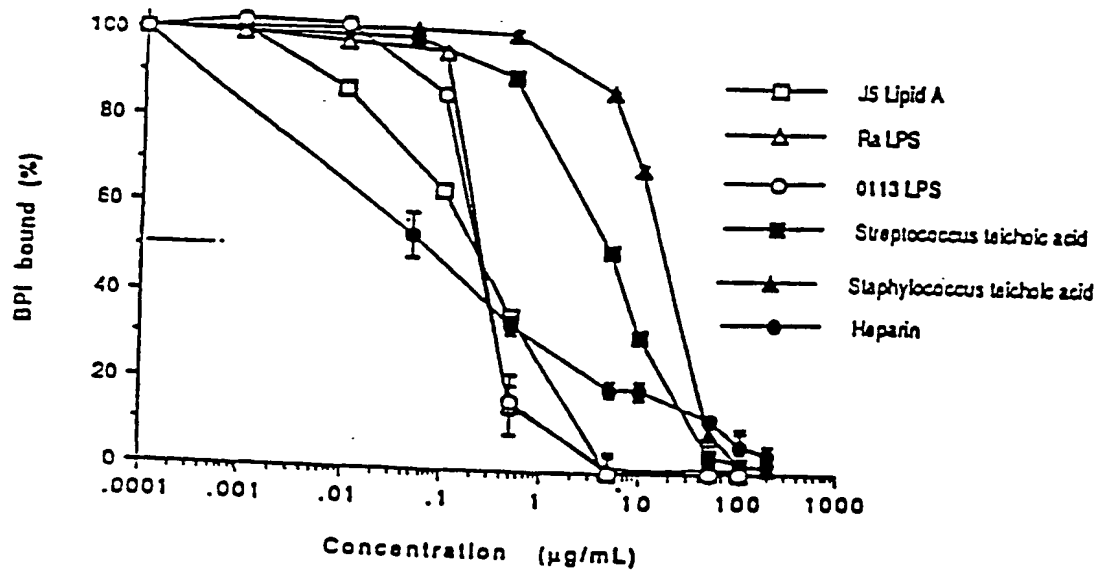


FIG. 2

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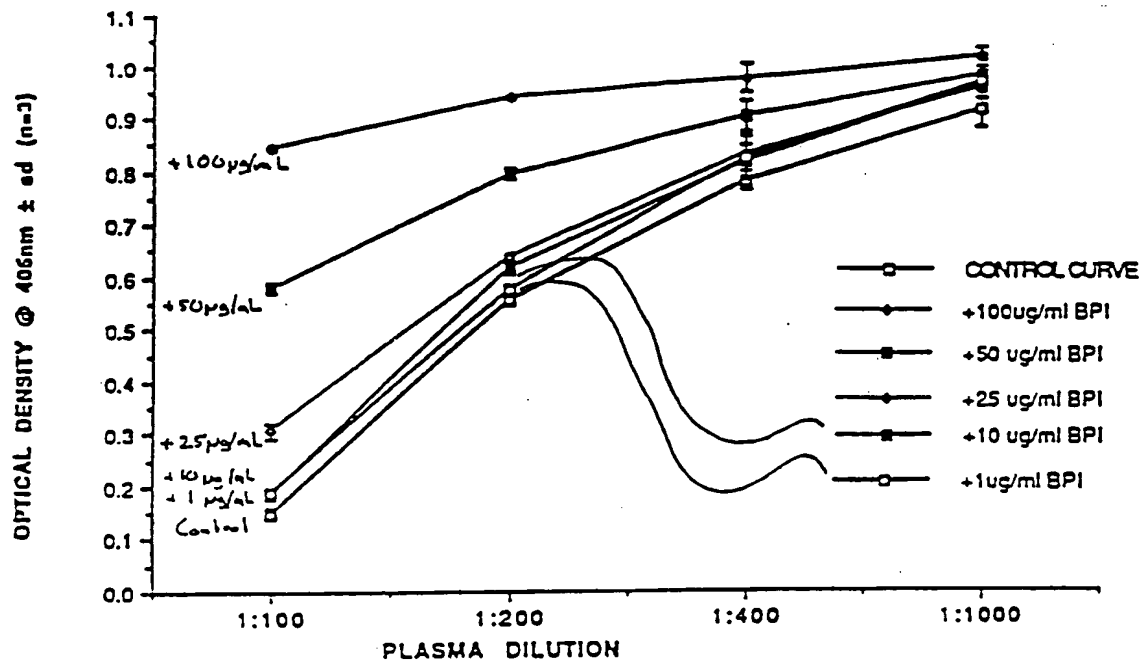


FIG. 3

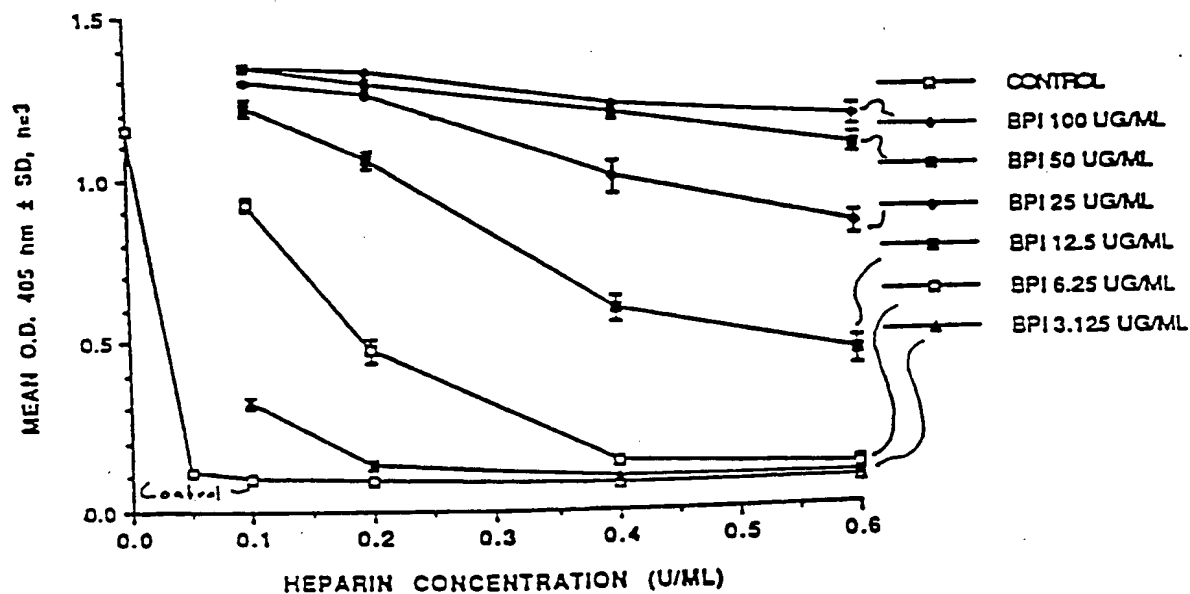


FIG. 4

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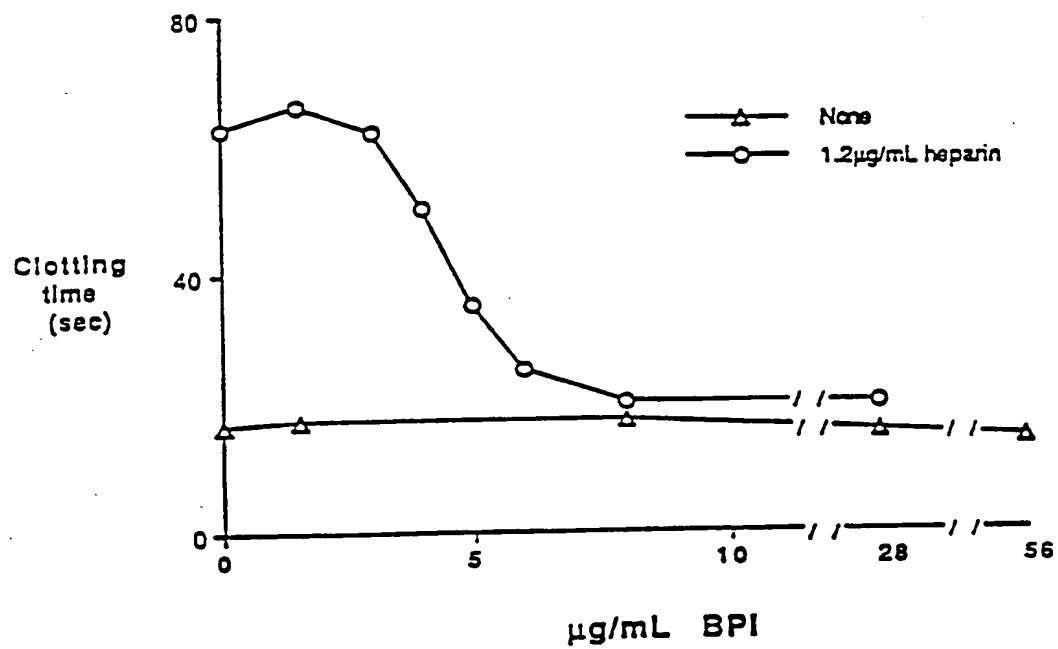


FIG. 5

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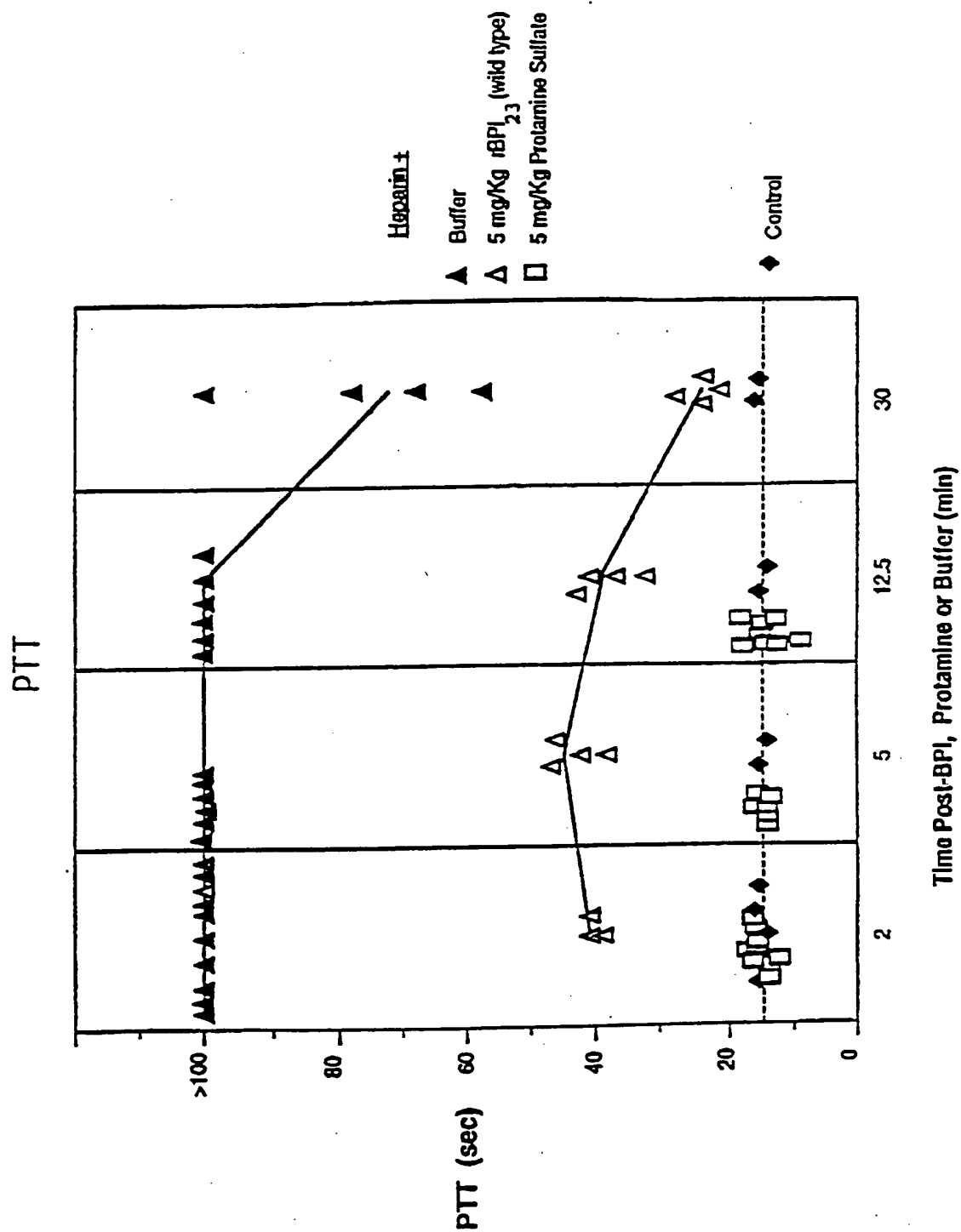


FIG. 6

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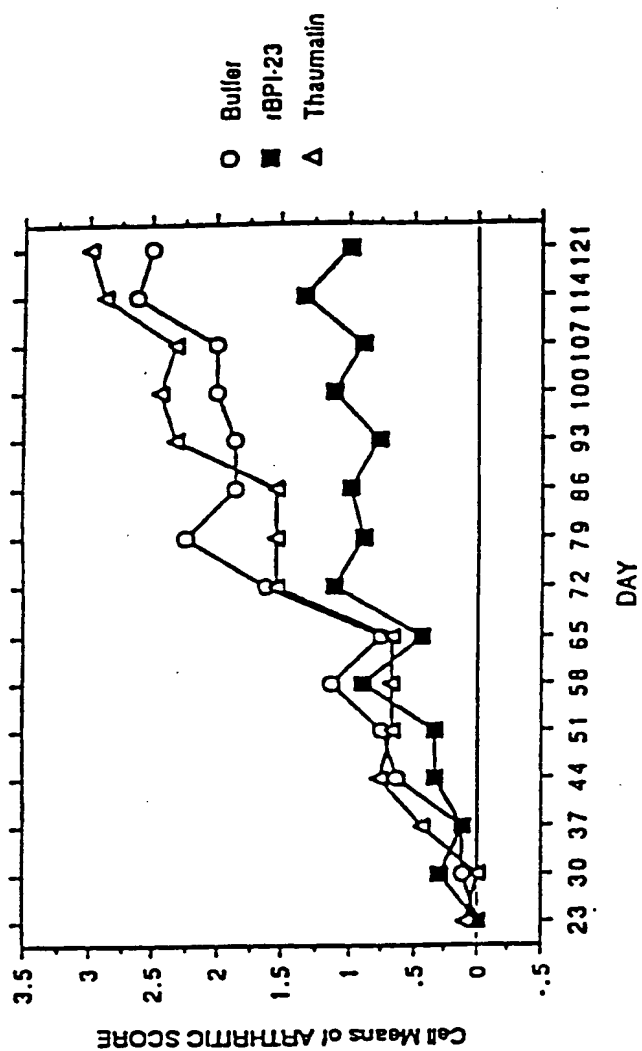


FIG. 7

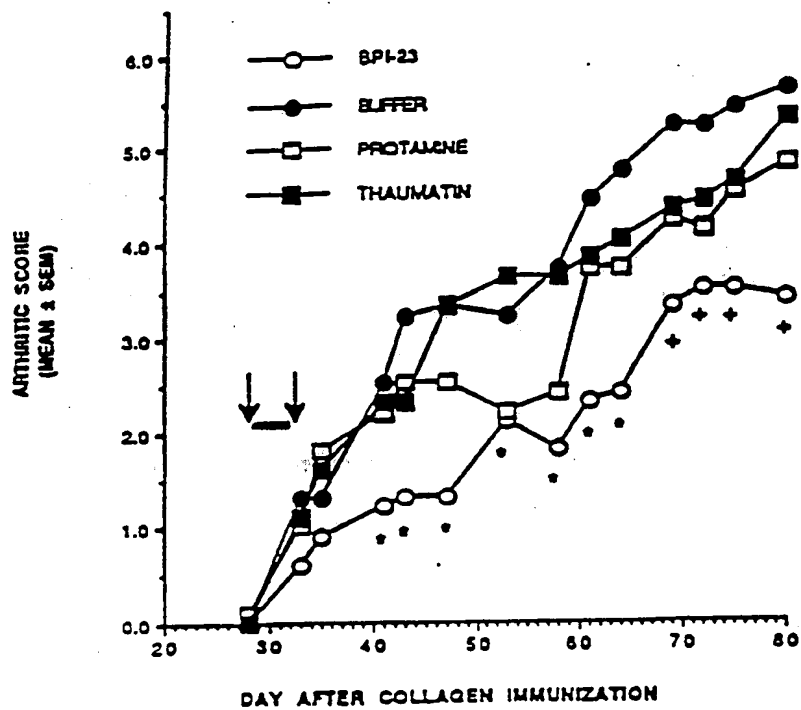


FIG. 8

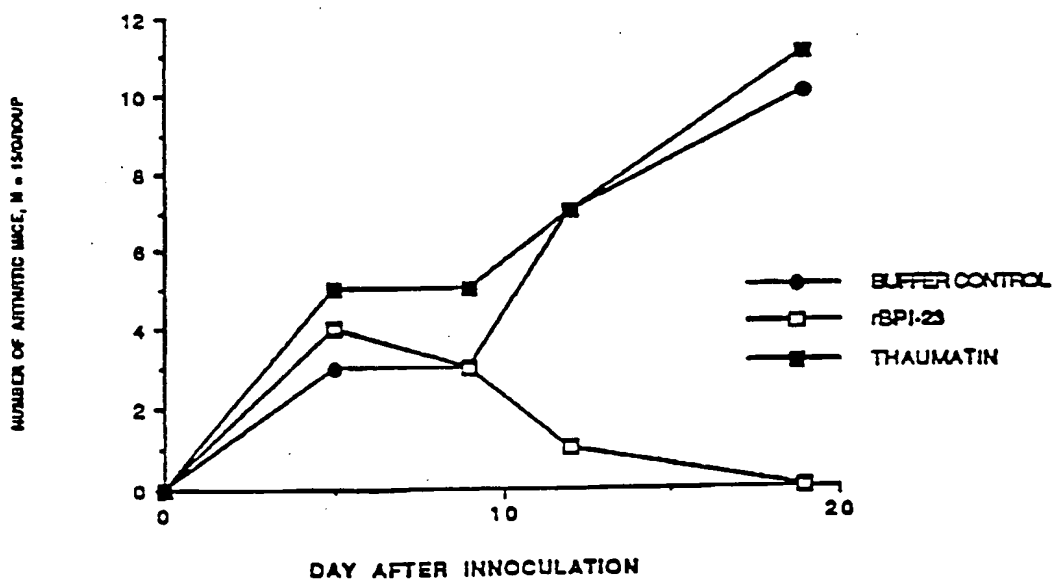


FIG. 9

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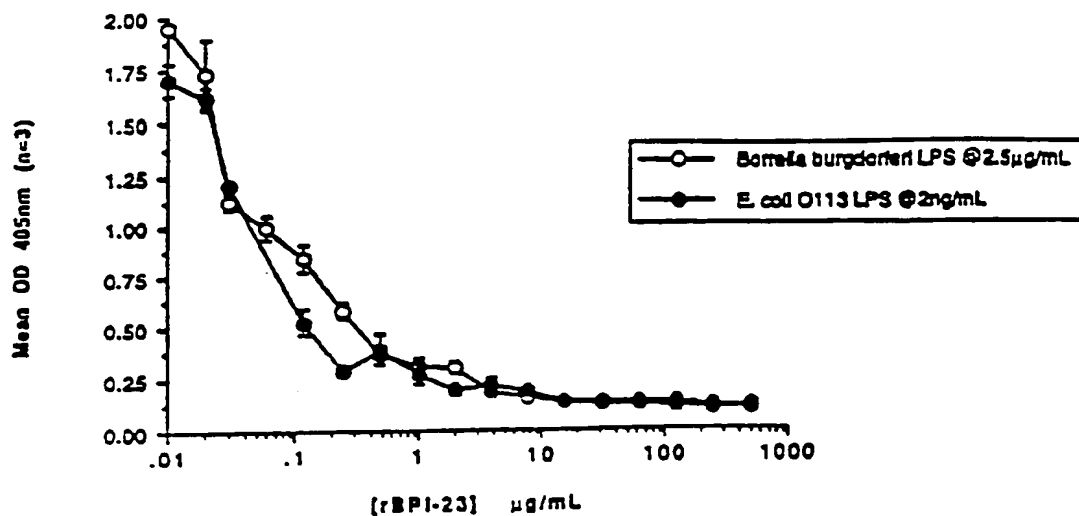


FIG. 10

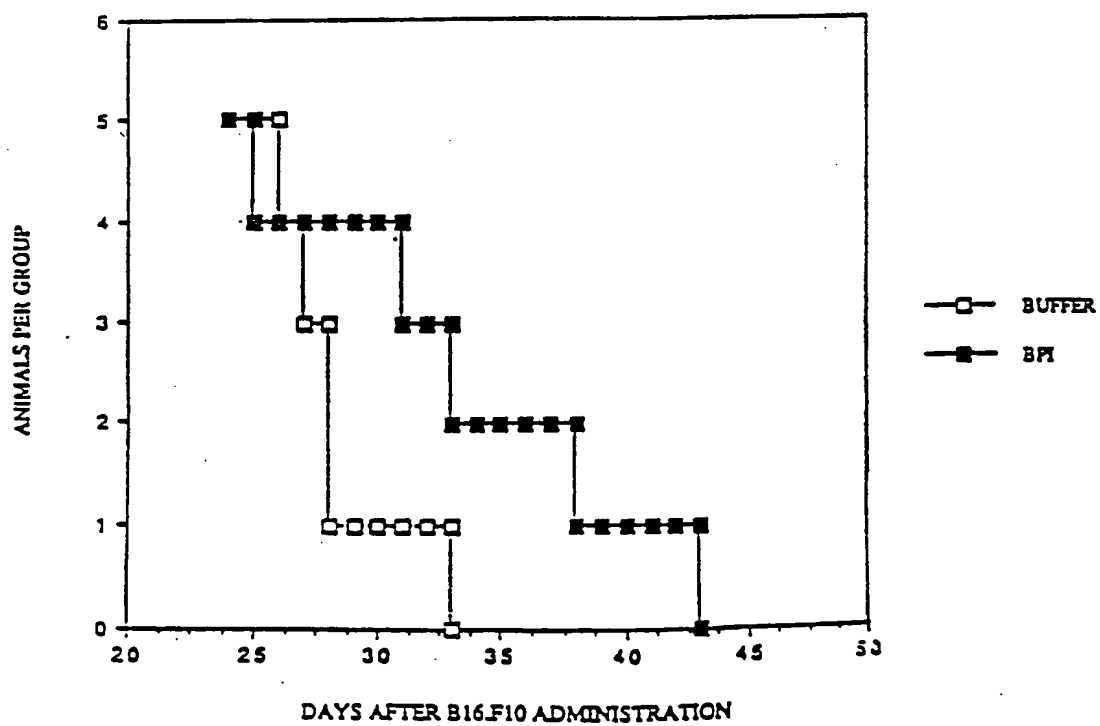


FIG. 11

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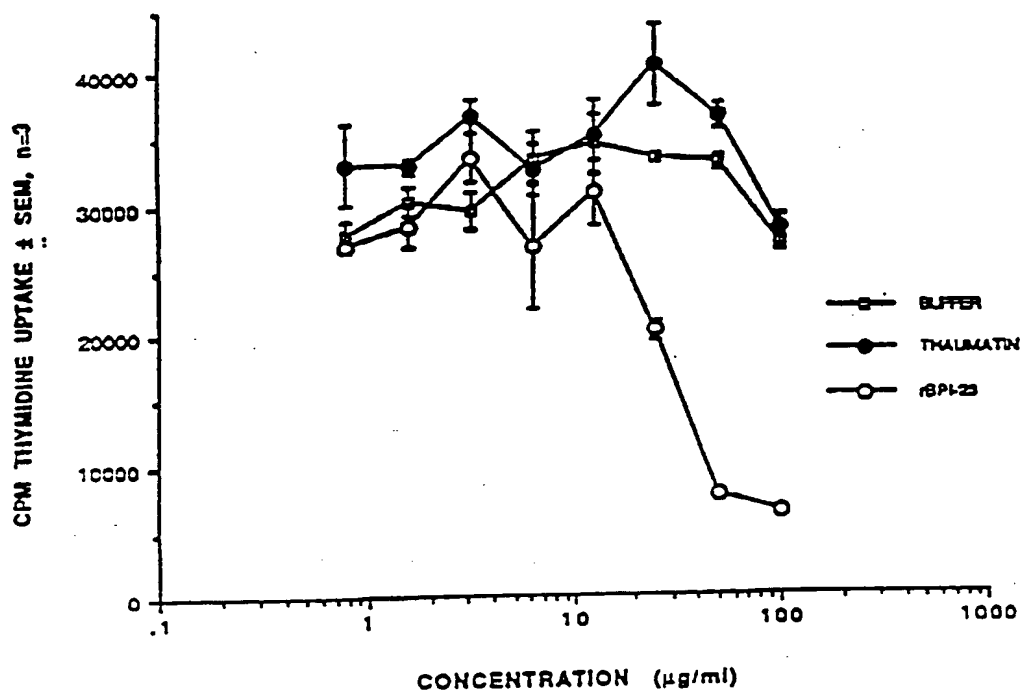


FIG. 12

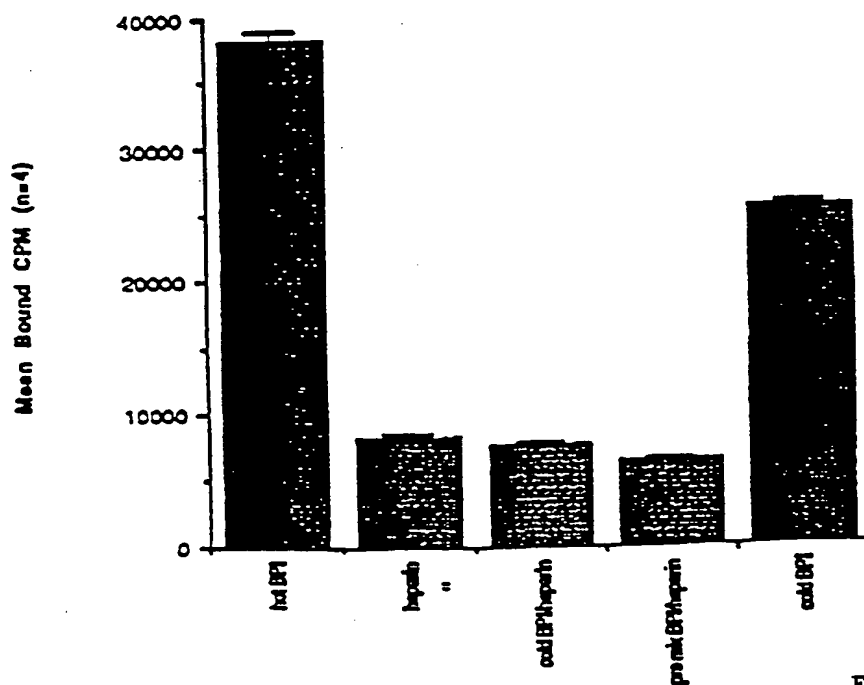


FIG. 13

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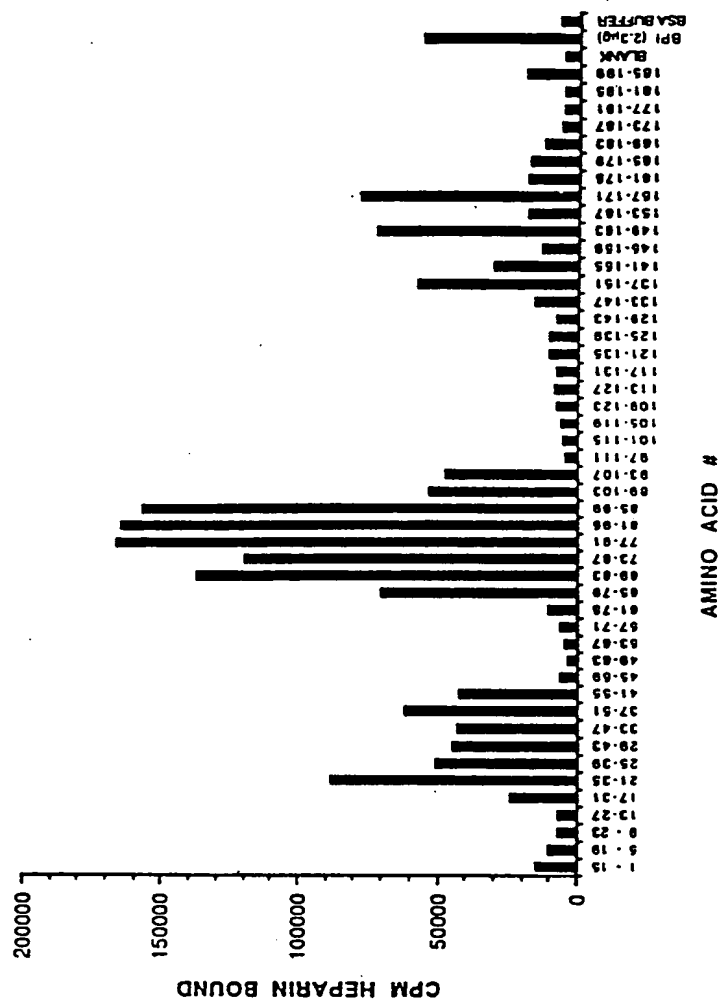


FIG. 14

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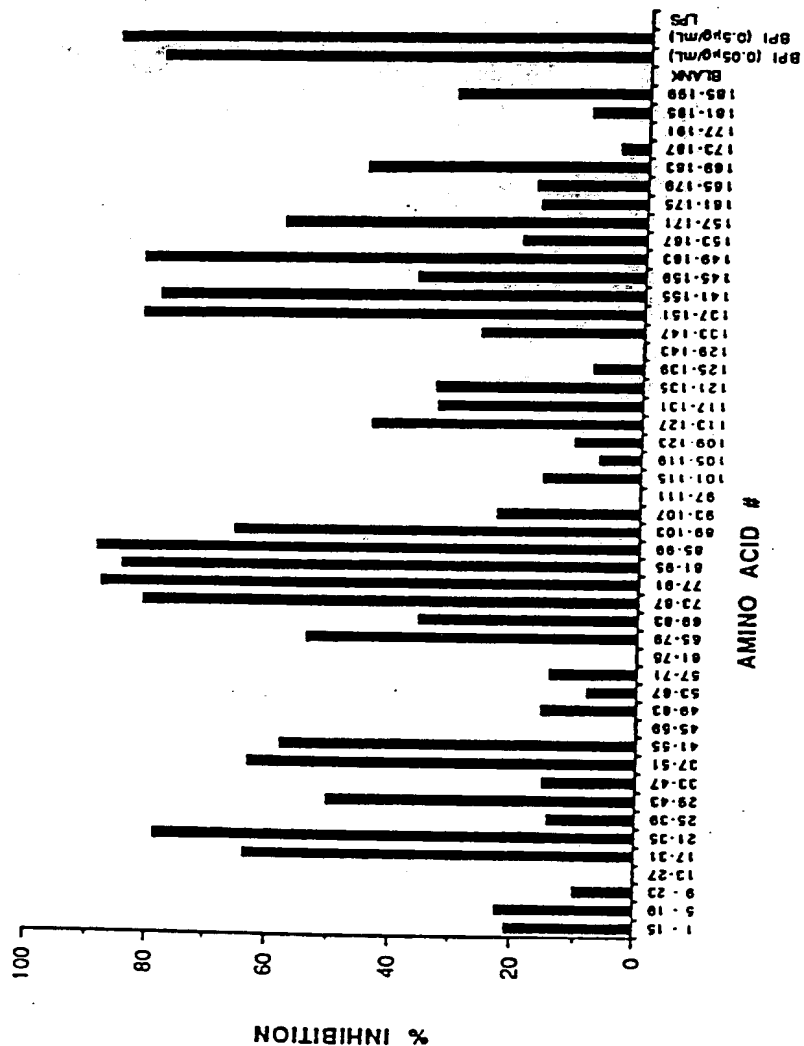


FIG. 15

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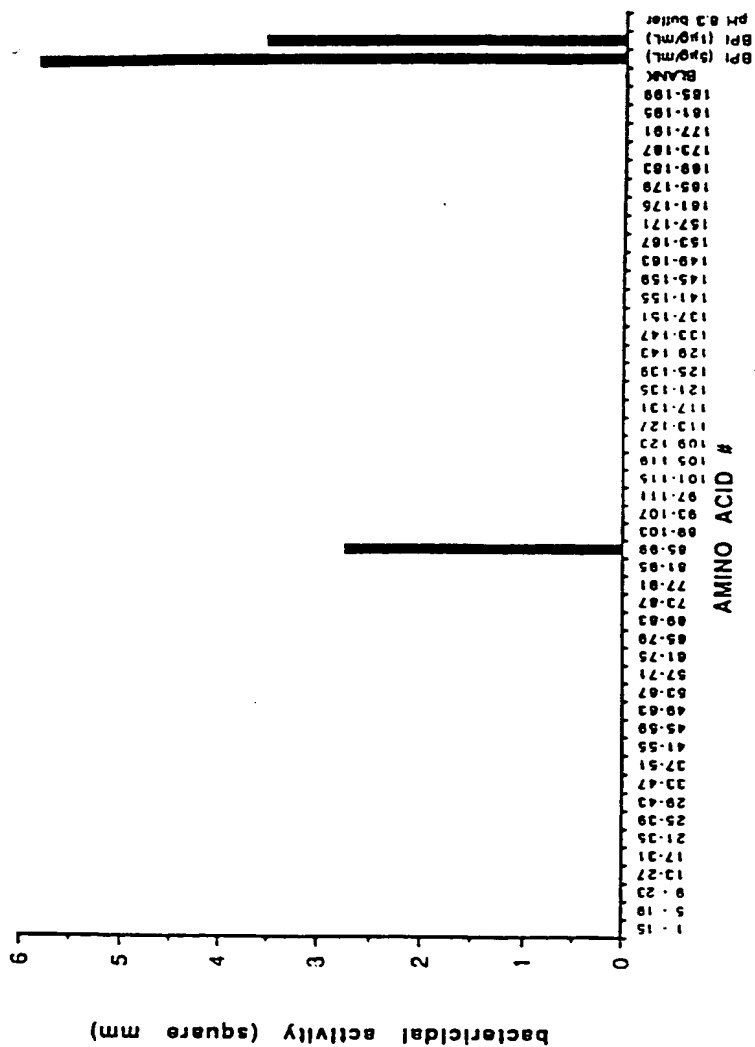


FIG. 16

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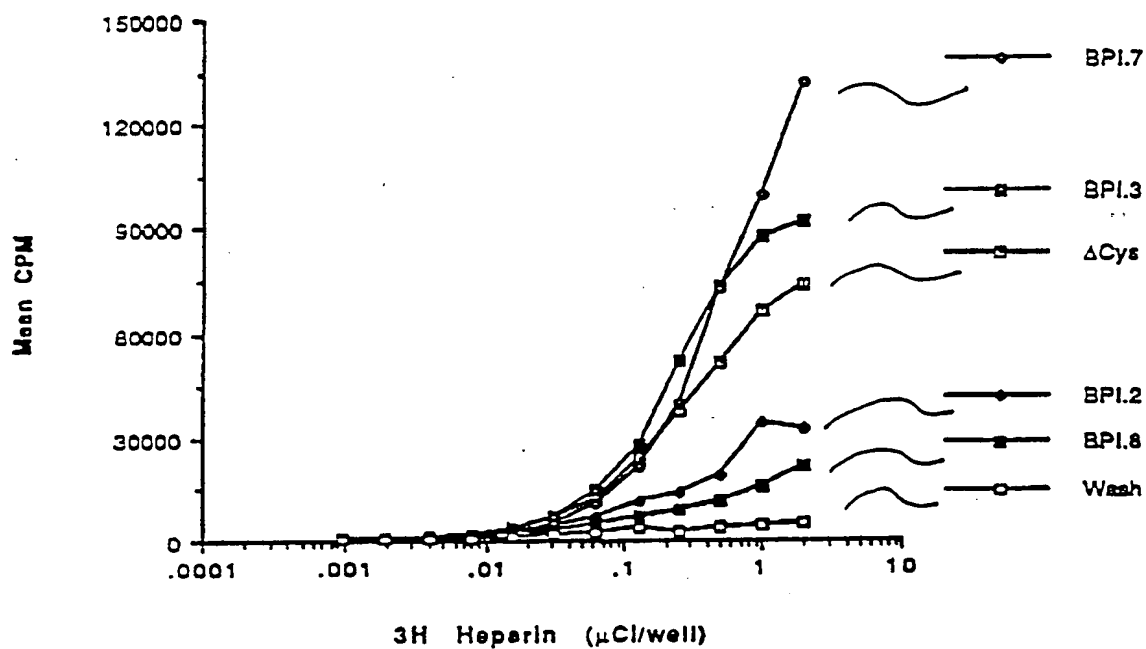


FIG. 17

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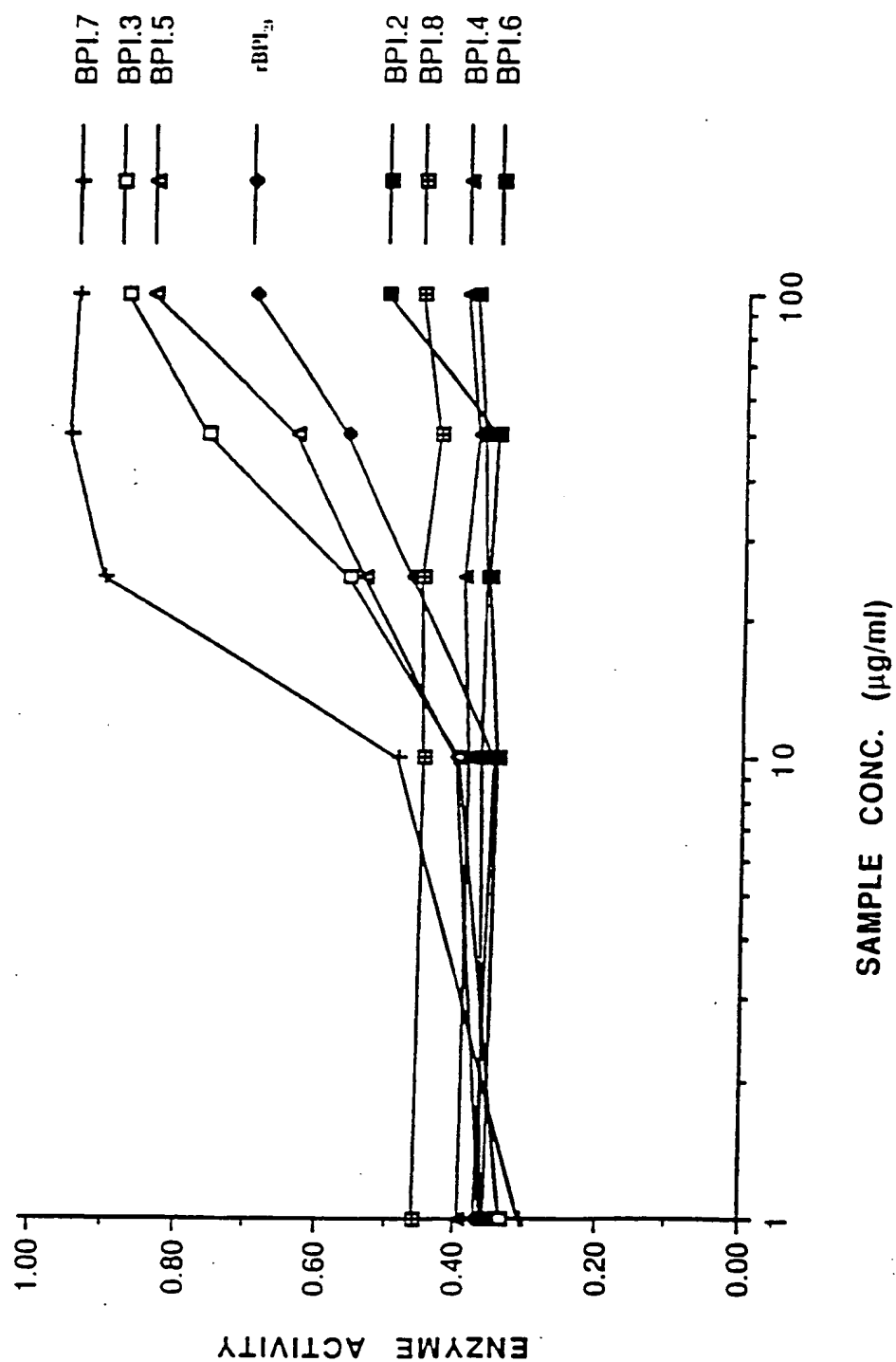


FIG. 18a

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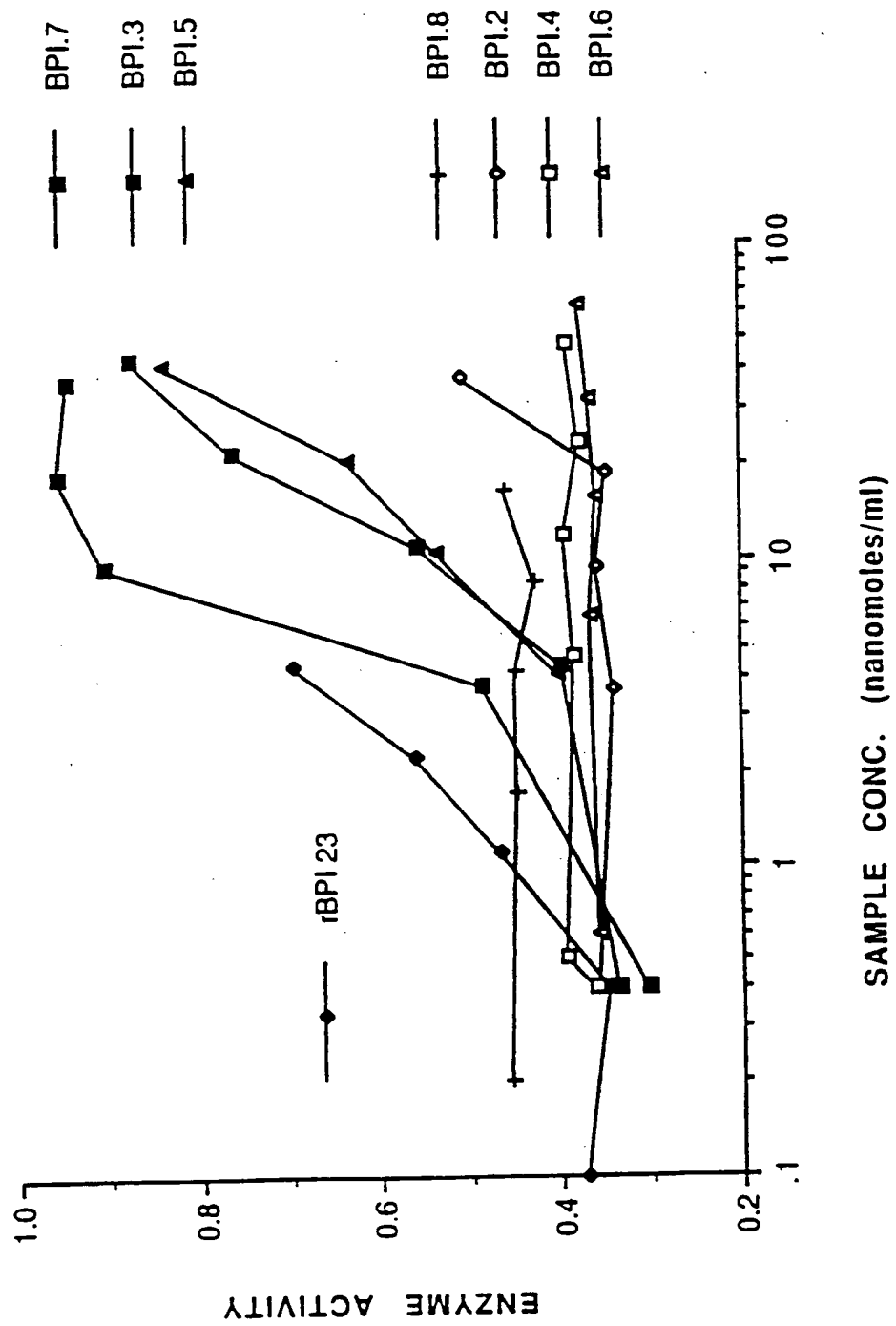


FIG. 18b

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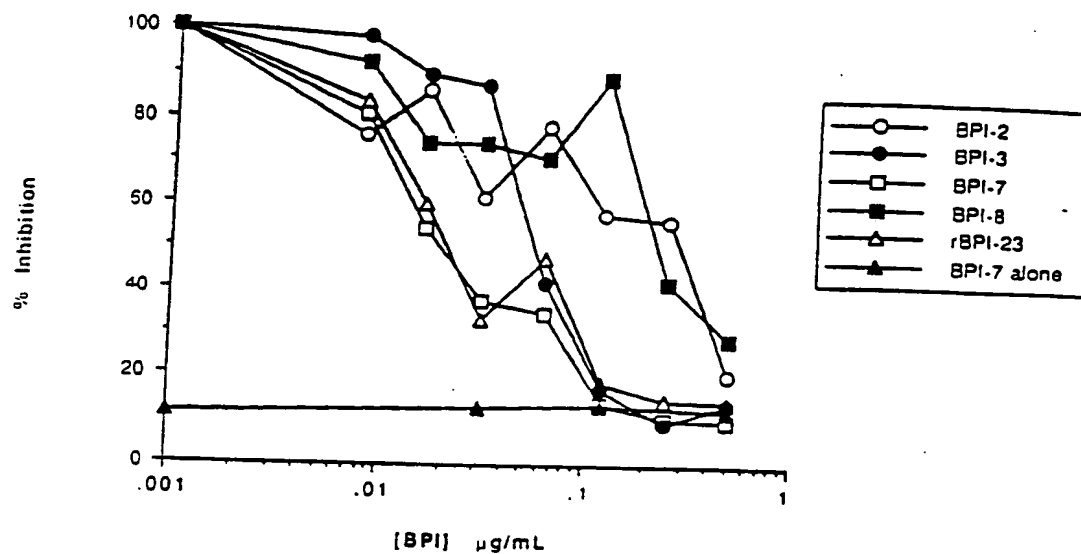


FIG. 19a

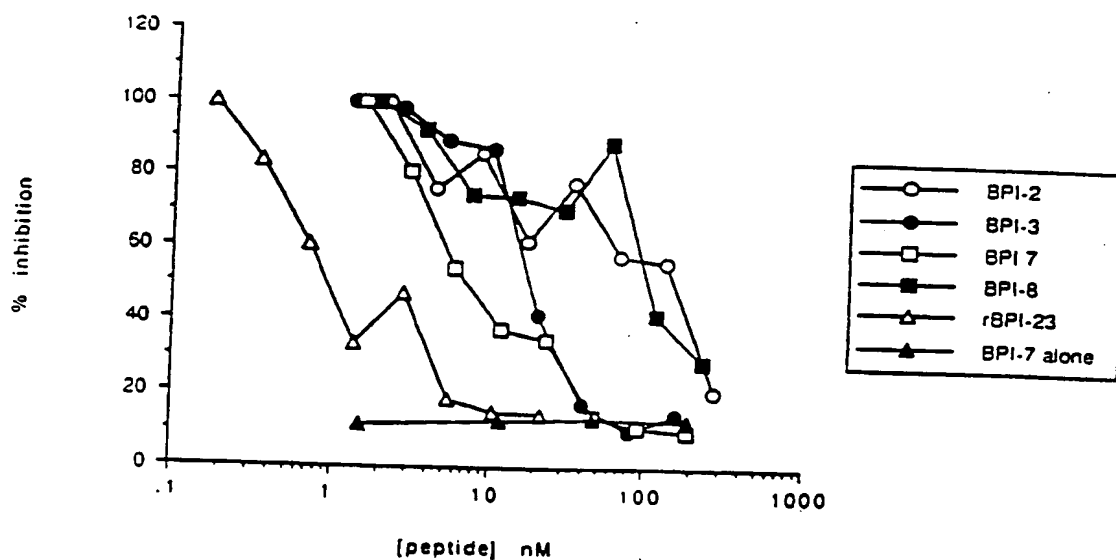


FIG. 19b

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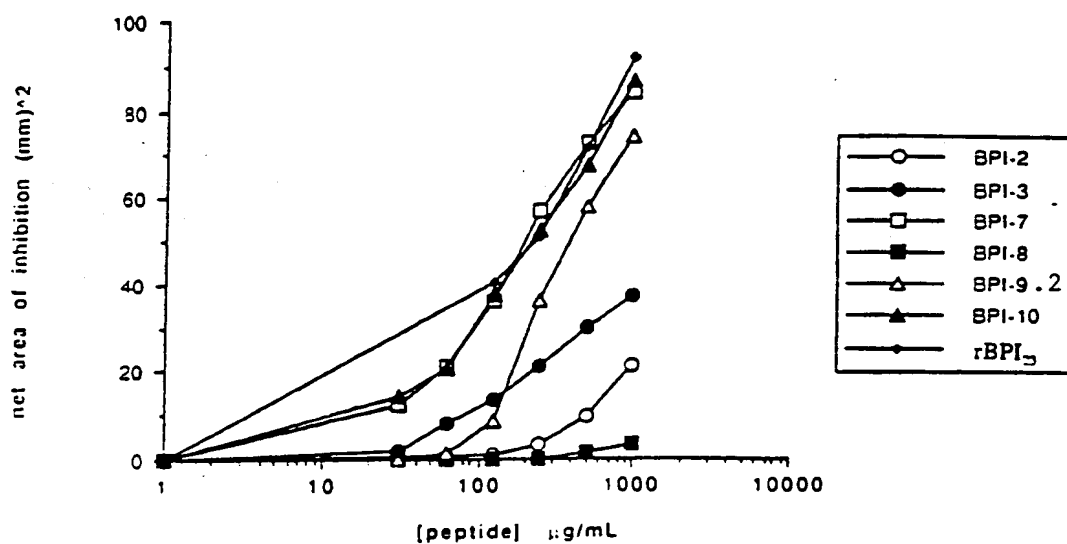


FIG. 20a

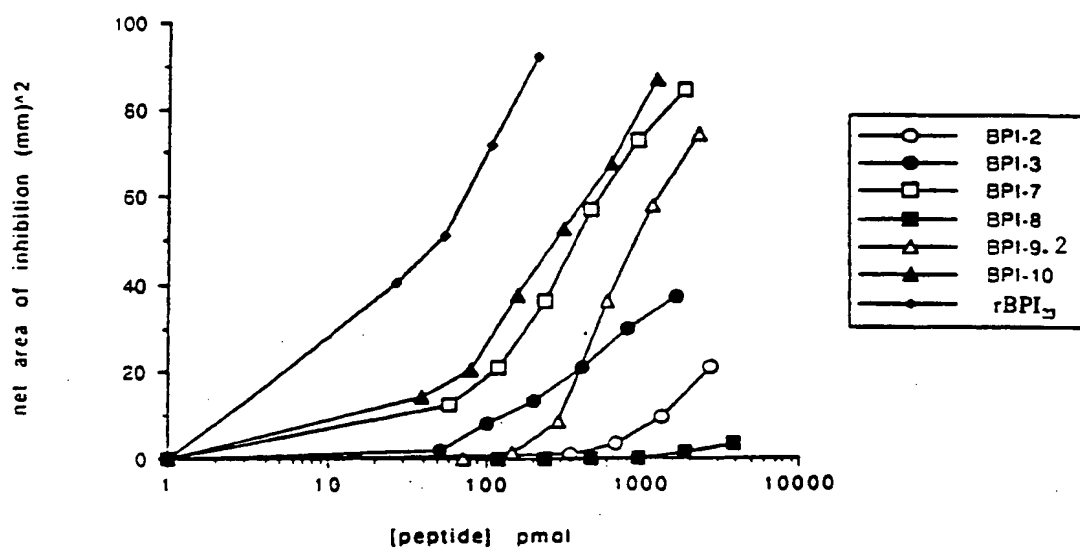
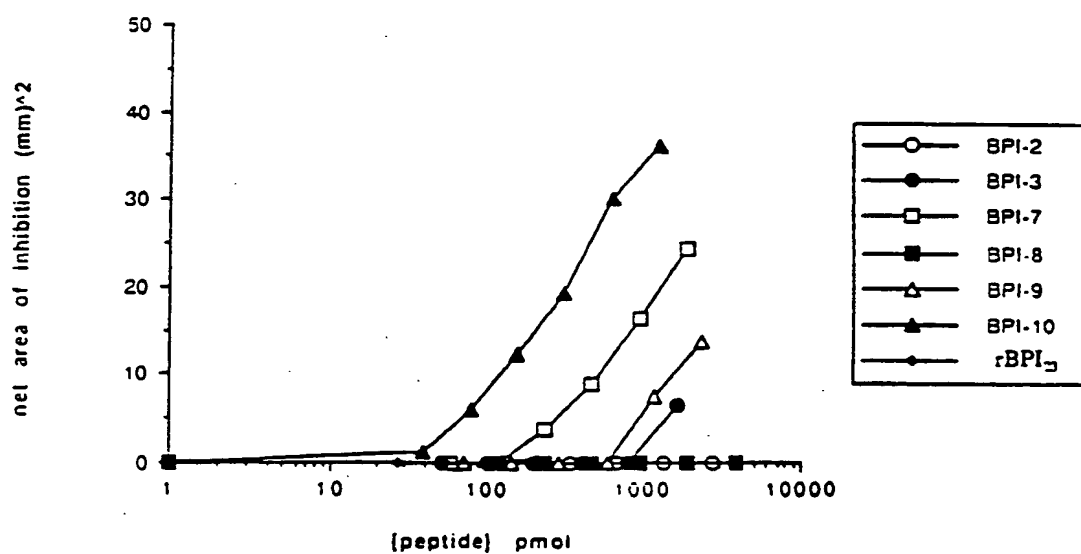
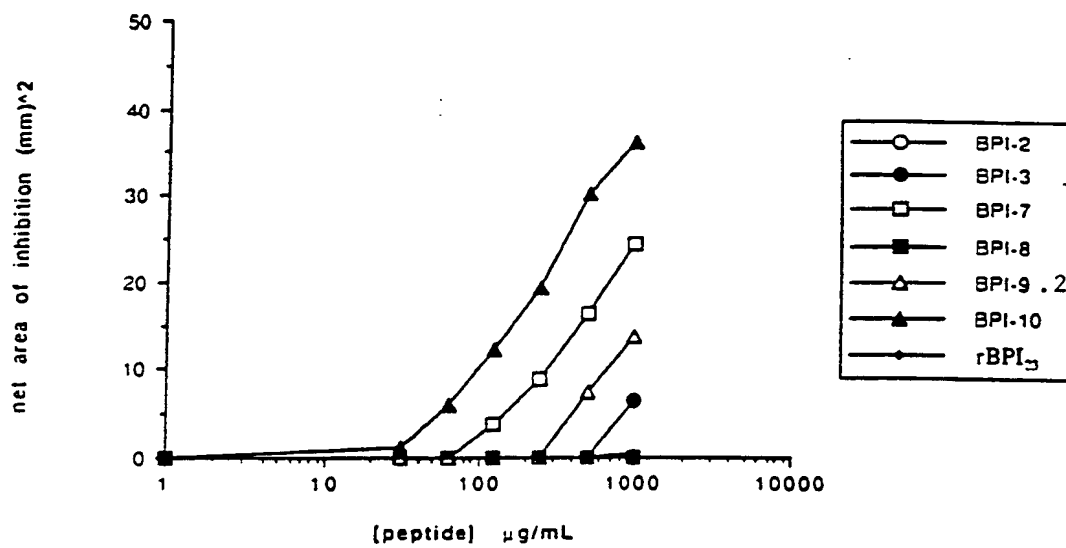


FIG. 20b

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SUBSTITUTE SHEET (RULE 26)

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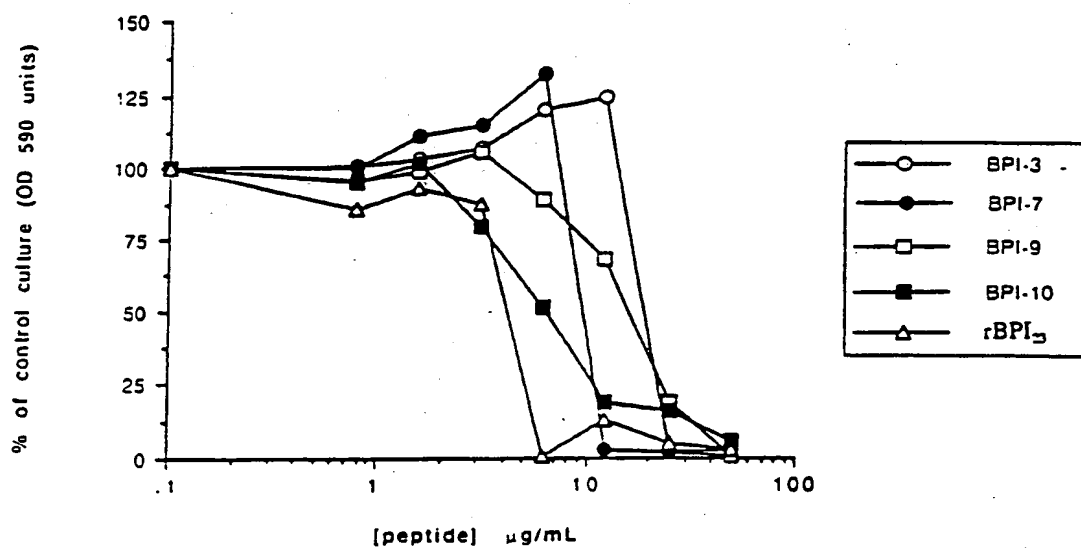


FIG. 20e

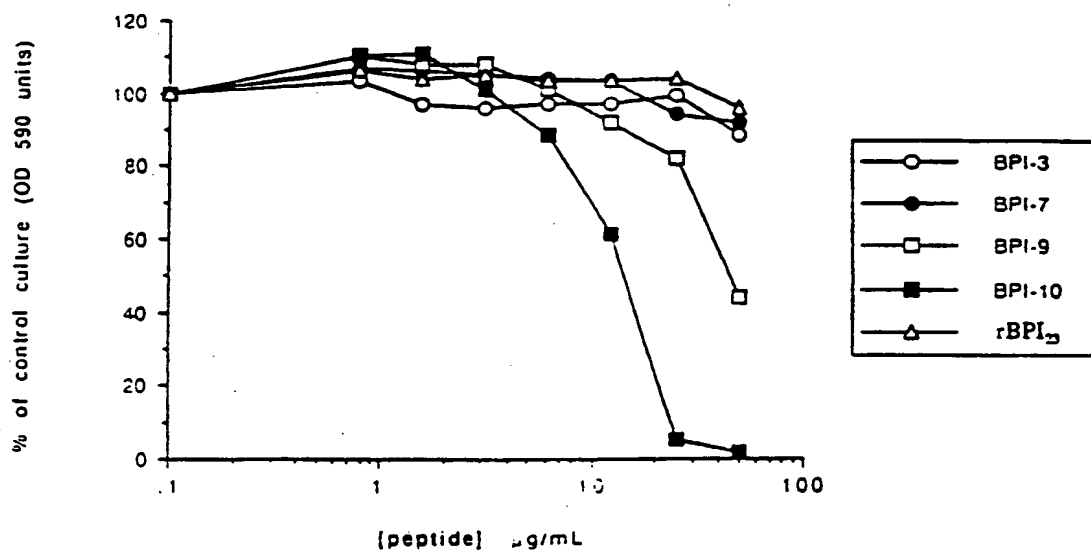


FIG. 20f

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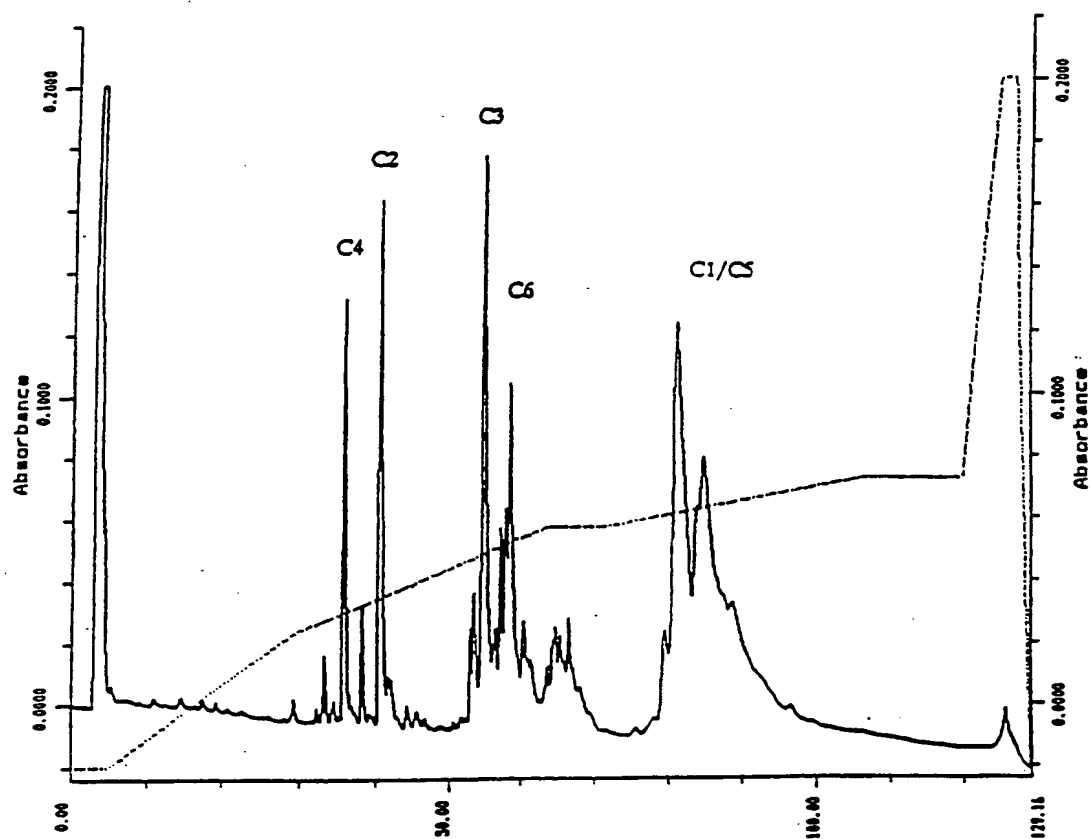


FIG. 21a

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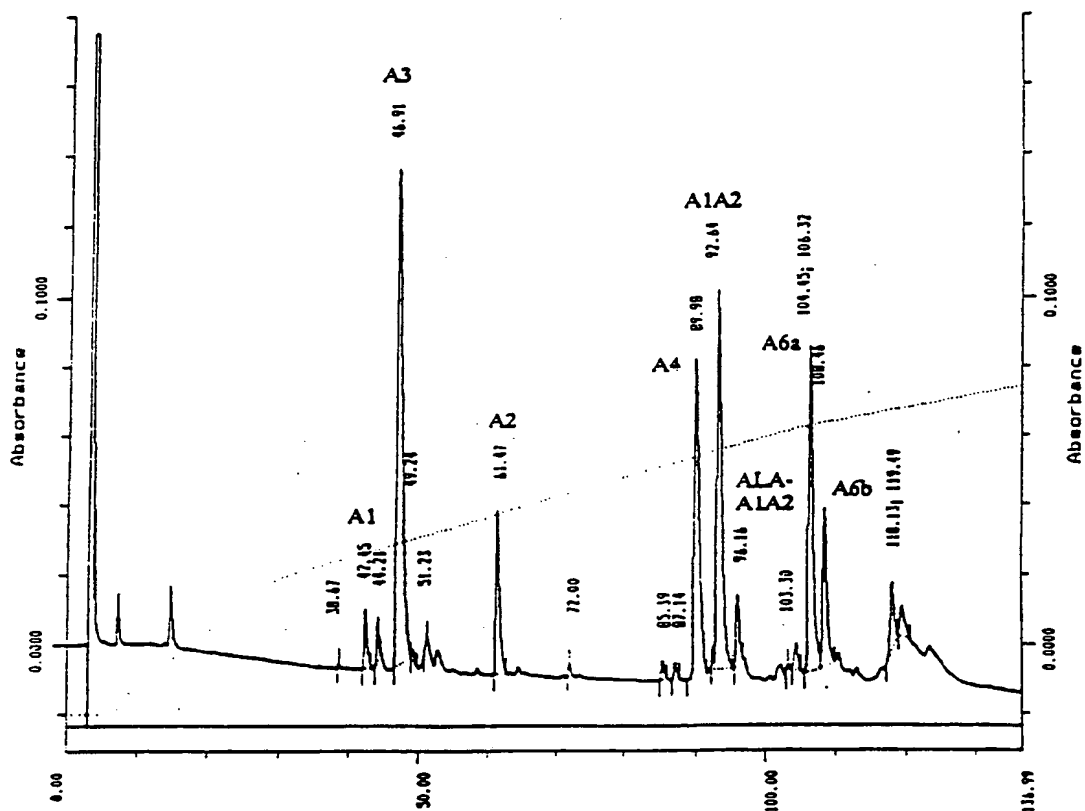


FIG. 21b

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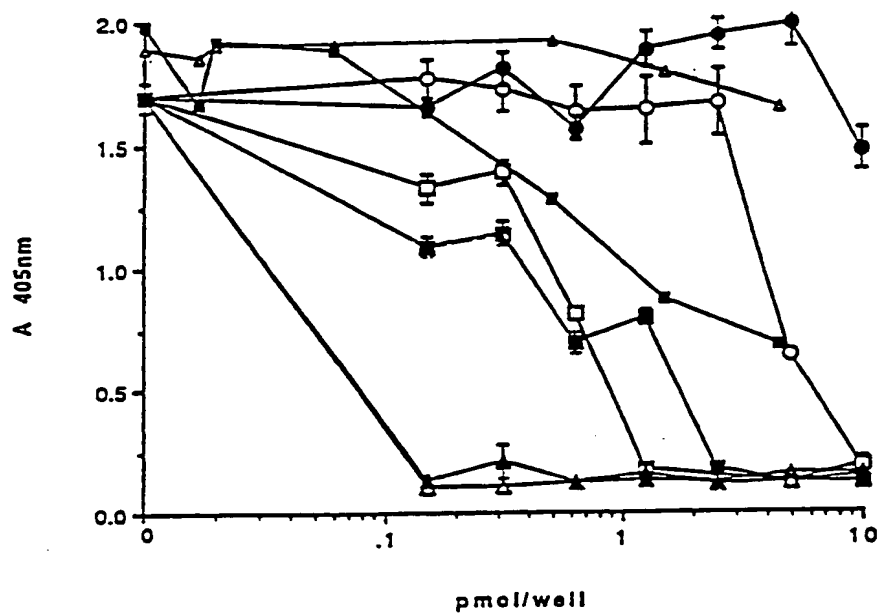


FIG. 22

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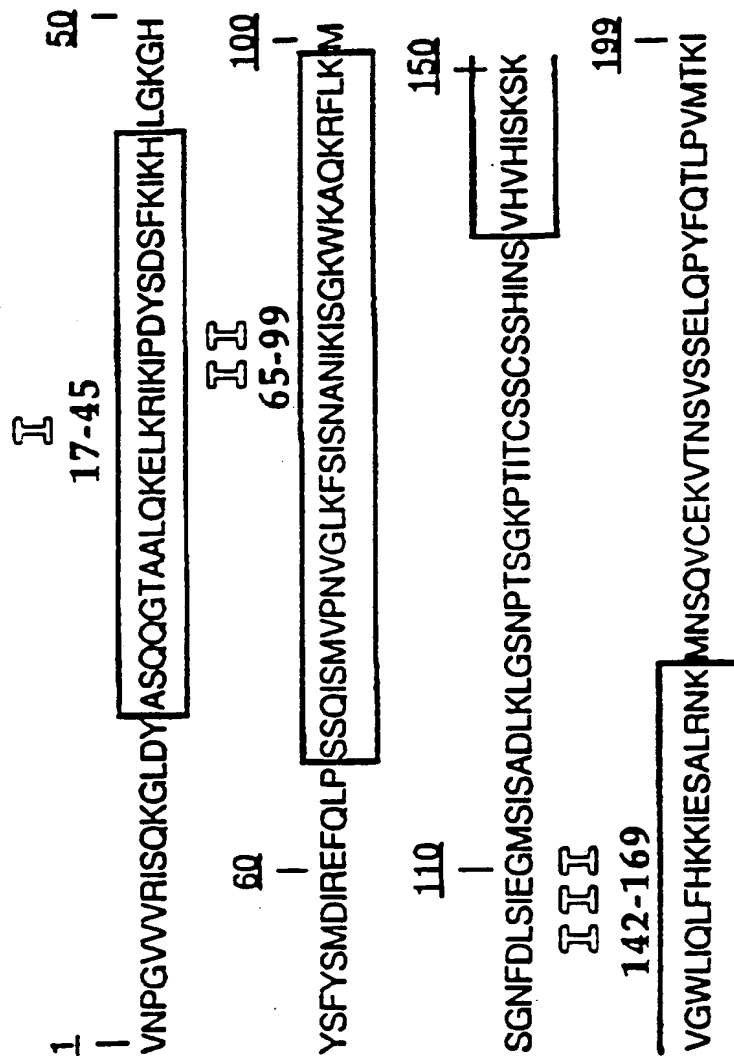


FIG. 23

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/02401

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K37/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,90 09183 (INVITRON CORPORATION) 23 August 1990 see the whole document ----	1-42
A	WO,A,92 09621 (NEW YORK UNIVERSITY) 11 June 1992 see the whole document ----	1-42
P,X	THE JOURNAL OF BIOLOGICAL CHEMISTRY vol. 269, no. 3, 21 January 1994, BALTIMORE pages 1865 - 1872 LITTLE R.G. ET AL. 'Functional domains of Recombinant Bactericidal/Permeability Increasing Protein (rBPI 23)' see the whole document -----	1-42

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- * 'A' document defining the general state of the art which is not considered to be of particular relevance
- * 'E' earlier document but published on or after the international filing date
- * 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- * 'O' document referring to an oral disclosure, use, exhibition or other means
- * 'P' document published prior to the international filing date but later than the priority date claimed

- * 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- * 'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- * 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- * '&' document member of the same patent family

Date of the actual completion of the international search

21 June 1994

Date of mailing of the international search report

12. 07. 94

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Moreau, J

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 94/02401

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 1-14 are directed to a method of treatment of the human/animal body (Article 52(4) EPC) the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No.

PCT/US 94/02401

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9009183	23-08-90	US-A- 5089274	18-02-92
		AU-B- 647734	31-03-94
		AU-A- 5170690	05-09-90
		EP-A- 0460058	11-12-91
		JP-T- 4506510	12-11-92
		US-A- 5171739	15-12-92
		US-A- 5234912	10-08-93
		US-A- 5308834	03-05-94

WO-A-9209621	11-06-92	AU-A- 9127591	25-06-92
		EP-A- 0563222	06-10-93

Form PCT/ISA/210 (patent family annex) (July 1992)